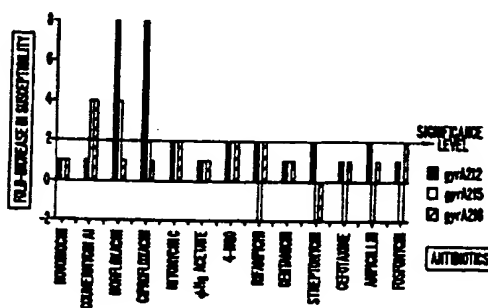




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(54) Title: SCREENING FOR MODULATORS OF BIOMOLECULES



(57) Abstract

This invention provides methods for screening for a modulator of a biomolecule by comparing the effects of a putative modulator on a phenotypic sensor in one or more conditional growth cell types having altered biomolecules with the effect on that sensor in a cell type in which those biomolecules are normal. The altered biomolecule or the biochemical pathways related to that biomolecule result in differential effects, and/or in phenotypic effect which can be essentially reproduced in the normal cell by the effects of the modulator. Also provided are methods for screening for a modulator and for characterizing and evaluating a modulator by comparing the effects of the modulator in a plurality of cells having different altered biomolecules and/or on a plurality of different phenotypic sensors. The methods are also applicable for characterizing novel modulators with reference to known modulators, such as to identifying a mechanism of action or characterizing the molecular interactions of the modulator.

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DESCRIPTION

SCREENING FOR MODULATORS OF BIOMOLECULES

Field of the Invention

This invention relates to methods for screening for enhancers or inhibitors of biomolecules such as enzymes or RNA molecules and the like, and to methods for characterizing such enhancers or inhibitors. Thus, in particular embodiments, this invention relates to the field of antimicrobial agents.

Background of the Invention

The following background information is not admitted to be prior art to the pending claims, but is provided only to aid the understanding of the reader.

The development of biologically active compounds for a large variety of purposes is of great interest in many different fields. As an example, in the field of antibacterial therapy bacterial resistance to current agents is a serious and increasing problem. The consequences of the increase in bacterial antibiotic resistance include longer patient hospitalizations and an increase in treatment costs. (B. Murray, 1994, *New Eng.*

J. Med. 330:1229-1230.) Therefore, there is great interest in the development of new antibacterial agents which are not affected by the existing bacterial resistance mechanisms, as well as agents providing improved treatment of infections caused by currently susceptible bacterial strains.

Similarly there is great interest in the development of compounds active against a variety of fungal organisms, including plant, animal, and human pathogens, as well as agents active on mammalian cells, such as anticancer agents.

The development of new therapeutic agents such as antibiotics can proceed by a variety of methods but generally fall into at least two categories. The first is the traditional approach of screening for antibacterial agents without concern for the specific target.

The second approach involves the identification of new targets, and the subsequent screening of compounds to find antibacterial agents affecting those targets. Such screening can involve any of a variety of techniques, including screening for inhibitors of the expression of a gene, or the function of a particular enzyme. While most such screening looks for growth inhibition of a particular wild type or resistant strain of an organism, certain methods utilize microbial strains which are hypersensitive to a particular class of inhibitor. Examples of such methods include Kitano et al., 1977, *Jap. J. Antibiot.* 30 Suppl.:S239-245, and Kamogashira and Takegata, 1988, *J. Antibiot.* 41:803-806, which report the use of bacterial strains hypersensitive to particular antibacterial agents for screening for additional inhibitors of the same class.

Another approach is reported in Andrianopoulos et al., 1995, *Identifying Biologically Active Agents Through Culture Color Change*, PCT Appl. PCT/US94/09304, Intern. Publ. No. WO 95/06132. That application reported the use
5 of mixed cultures of two organisms, each of which produced a different color culture, and one of which is more sensitive toward a particular class of inhibitor. Preferential inhibition of one of the sensitive strain causes the mixed culture to exhibit the color of the other
10 strain, and suggests the presence of a compound active against a particular target. Yet another approach is described in C. Selitrennikoff, 1983, *Antimicrob. Agents Chemother.* 23:757-765, in which the regeneration of the cell wall of a *Neurospora crassa* mutant strain was
15 monitored to detect antifungal compounds which may act on fungal cell wall assembly functions.

Summary of the Invention

This invention provides methods for rapid screening and characterizing of modulators of biomolecules
20 in a sensitive, specific, and highly informative manner. Specifically, these methods allow screening for modulators of biomolecules, including screening for antimicrobial agents, by the use of cell types which exhibit conditional growth phenotypes. Such mutant cell types are more
25 susceptible to agents that act on the mutated biomolecule or which amplify the defect caused thereby, thus providing an indication of useful modulators based on differential growth of the mutant and normal cell types in the presence of such a modulator. Examples of such mutants include
30 temperature-sensitive mutants. In addition, other

phenotypic sensors, such as differential carbon source utilization by mutant strains, can be utilized to screen for or characterize a modulator. The biomolecules which may be mutated include, for example, enzymes such as DNA
5 gyrase and RNA activators. Further, the invention allows rapid screening of a number of different biomolecules simultaneously.

In addition, the invention provides methods for screening, evaluating, and characterizing a modulator of
10 a biomolecule or a preparation which contains such a modulator using a multichannel approach. These methods thus allow simultaneous determination of a broader range of relevant biological characteristics at an early stage of compound screening than is provided by traditional
15 screening approaches. In some of the methods of this invention, a compound or preparation is evaluated using a variety of different assays simultaneously, and can therefore provide information such as whether a compound inhibits growth of a particular cell type, the target(s)
20 of the compound, relevant toxicological properties, and relevant pharmacological properties. Such pharmacological properties include, for example, serum binding, serum inactivation, solubility, stability, and breadth of spectrum of activity (across different cell types). Taken
25 together, the results of the set of assays in a multichannel screen provide an identifiable pattern characterizing the mechanism of action of a biologically active compound, and in some cases, even characterizing the compound itself.

30 Thus, in a first aspect the invention features a method for screening for a modulator of a biomolecule.

The method includes determining the effects of a potential modulator by comparing a phenotypic sensor between a first cell type having an altered biomolecule and a second cell type having a normal biomolecule. The cell type having an altered biomolecule has a conditional growth phenotype, or the altered biomolecule has a partially crippled function. At least one of the first and second cell types are grown in contact with a potential modulator in an appropriate growth medium, preferably under semi-permissive conditions, such that the function of the altered biomolecule is partially crippled. Preferably the first and second cell types are separately in contact with the potential modulator.

The term "phenotypic sensor" refers to an observable characteristic of a cell type which is characteristic of the metabolic functioning of the cell. Thus, a phenotypic sensor is an indicator of differences in the function of particular biomolecules within a cell, and will differ between two cell types which have different functional levels of a particular biomolecule. Such a phenotypic sensor may be a dynamic characteristic, such as the ability to grow under certain conditions, or a cellular status, such as the intracellular level of certain metabolites. The sensor can vary depending on the conditions under which the determination is performed and/or the cells grown.

By "screening" is meant that the previously unknown properties of interest of a molecule are determined in the assay. This procedure is distinct from an individual test to determine the properties of such a molecule. Generally, the method includes screening of a

large number of potential modulators simultaneously, for example, 5 or 50 or more such modulators. Those in the art will recognize that potential modulators include a wide variety of biochemical molecules including small
5 molecules of molecular weight less than three thousand, as well as larger molecules including oligonucleotides, peptides, lipids, and carbohydrates.

A "modulator" or "modulator of a biomolecule" or "biomodulator" is an agent which is able to affect the
10 activity of a biomolecule by either inhibiting or enhancing that activity. Generally, such a modulator is an inhibitor of the biomolecule. Thus, modulators include, for example, antimicrobial agents and anticancer agents. A "known biomodulator" is a compound which is
15 known to be biologically active on particular cell, but the particular mode of action or cellular target need not be known. A "potential modulator" is a test compound is a screen or evaluation.

By "comprising" it is meant including, but not
20 limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

By "biomolecule" is meant any molecule that is
25 normally present within a living cell or organism which is produced by or utilized by that cell or organism, including proteins, peptides, polypeptides, carbohydrates, lipids, RNA, DNA, and oligonucleotides.

An "altered biomolecule" is one that differs from
30 that present in the naturally occurring microbe, i.e., a normal microbe. By "altered" is meant that the

biomolecule is either defective in its activity, that is, it has a reduced level of activity (e.g., 20% reduced, but preferably not completely diminished activity), or has an enhanced activity, that is, an activity greater (e.g., at least 25% more) than that found in the normal biomolecule. The term also includes a defect in amount, e.g., overexpression or underexpression of the biomolecule. Examples of such biomolecules include DNA gyrases as exemplified below. Applicant has determined that such altered biomolecules are more sensitive to agents which act at those molecules, for example, a DNA gyrase is more susceptible to fluoroquinolones. Such greater susceptibility allows more sensitive detection of agents, i.e., modulators, which act at those biomolecules. Thus, applicant has determined that potential modulators of a particular enzyme can be readily screened in a rapid assay for activity at such molecules using microbes with defective biomolecules. The term "different altered biomolecules" indicates distinguishable alterations of biomolecules. This can, for example, include alterations to biomolecules of different types and/or phenotypically distinguishable alterations to biomolecules which are the same except for that alteration.

The term "cell type" includes both haploid and diploid cells. For example, diploid cells can include mammalian, e.g., human, immortal cell lines and diploid microbial cells. The term "cell type" refers to a particular strain or cell line.

The term "conditional growth phenotype" means that the growth of the cell type having that phenotype is conditional on at least one variable of the culture

conditions to a greater degree than a normal cell type. Generally, the comparison is between a normal parental cell type and a conditional growth derivative of the parent. Thus, the conditional growth cells will exhibit
5 greater difference in growth between permissive conditions and restrictive conditions than normal cells, and will grow more slowly or with detectable phenotypic differences under semi-permissive (intermediate) conditions. A particular example of such a conditional growth phenotype
10 is a temperature sensitive phenotype. Typically, in comparison to a normal cell type, growth of a temperature sensitive cell type, e.g., a bacterial strain, will be inhibited at some elevated temperature, while having similar growth at a lower temperature. Thus, "conditional
15 growth" refers to the differential response of the cell type to a change in some culture condition variable as compared to a normal cell. Examples of such culture condition variables include, but are not restricted to, temperature, salt concentration, pH, and nutrient
20 utilization. It is not intended to refer to merely a differential response to the presence of some concentration of anti-growth agent, e.g., antimicrobial agents, although as described below in the Detailed Description and Example, conditional growth cells commonly
25 exhibit hypersensitivity to one or more anti-growth agents when grown under semi-permissive conditions (for example, the increased susceptibility of a bacterial strain to an antibacterial agent). However, cell types which are hypersensitive to a compound but do not otherwise exhibit
30 a conditional growth phenotype are useful in the multi-channel methods described herein.

"Partially crippled function" means that a biomolecule has lowered biological activity under normal cellular conditions than a normal copy of that biomolecule.

5 In the context of cells and cell types the term "growth" refers to an increase in the number of cells. For particular cell types the term may also include an increase in the size of individual cells particularly an increase preparatory to cell division. Thus, "comparing
10 growth" means determining whether one cell type exhibited a greater or lesser change in cell numbers and/or size than in other cell type or whether a particular cell type exhibits greater or lesser growth under one set of conditions than under another set of conditions. A direct
15 or indirect measure of such growth can be used. For example, the turbidity of a medium can be monitored by standard procedures, the pH of the medium monitored, or the viability of cells monitored with a fluorophore. Those in the art will recognize that other direct or
20 indirect methods of measuring growth of the cells are within the scope of this invention. More generally, "comparing a phenotypic sensor" means determining the differences or similarities for the observable characteristic for two or more different cell types and/or
25 conditions. In comparing patterns of growth or carbon utilization or phenotypic profiles, the term "comparing" means that the overall body of test results is considered. In some cases it is possible to compare result patterns with only simple analysis and display, such as simple
30 calculation of results and graphing. However, in many applications, the amount of data and the types of data

involved will make the use of statistical analysis and computer based pattern recognition highly beneficial. Some possibilities for such analysis are described in the Detailed Description.

5 The partially crippled biomolecule is more susceptible to the action of a modulator which acts on the altered biomolecule or a biochemical pathway related to that biomolecule. Such a modulator thus causes differential effects on a phenotypic sensor (such as
10 differential growth) of the first and second cell types when both are grown in contact with that modulator. Therefore, a screen result showing a compound which results in a greater effect on the cell type having the altered biomolecule than on the cell type in which that
15 biomolecule is unaltered is an indication that the compound is a modulator targeting that particular biomolecule or a related biochemical pathway. Therefore, in a preferred embodiment, the method involves growing the first and second cell types in contact with a potential
20 modulator. A modulator which acts on the altered biomolecule or a related biochemical pathway results in a greater difference in the phenotypic sensor when the modulator is present than when it is absent. In particular, the phenotypic sensor can be growth.

25 The use of a plurality of different cell types having different altered biomolecules provides a broader range of comparison information, therefore, in a preferred embodiment, a plurality of first cell types is used. Such a plurality may be a small number, e.g., two or three, but
30 preferably is larger in order to include a large number, e.g., 50 or 100 or more, of biomolecular alterations and

other cell type differences. The plurality of resulting phenotypic sensor comparisons thus can provide both a broader range of information on a particular modulator and a broader scope to a screen by monitoring the effects on
5 a large number of different targets.

In another preferred embodiment, the cell type having the normal biomolecule (second cell type) is grown in the presence of the potential modulator, and the comparing involves determining whether the phenotypic
10 sensor is the same for the different cell types. This is based on the observation that the cellular effects of a particular altered biomolecule can be essentially reproduced in a wild type (normal) cell by the action of a modulator which targets that biomolecule or a
15 biochemical pathway related to that biomolecule.

In the context of comparisons of phenotypic sensors or patterns, "same" means a characteristic degree of similarity which functionally distinguishes a pair of observations or patterns from other less related pairings.
20 Thus, the term does not mean identical; differences of type or degree may exist between the members of a pair. For example, in comparing patterns of carbon utilization for a normal cell in the presence of a modulator and a mutant cell, the amount of growth of the two cell types in
25 culture media differing in their carbon sources can differ, though the patterns could still be the same. Other phenotypic sensor and pattern comparisons should be considered in a similar manner.

As suggested above, the cell types are preferably
30 grown under a plurality of different growth conditions. Therefore, in particular embodiments, the comparing is

performed in a number of different growth conditions. This can provide a pattern over the different growth conditions which is useful for comparison, and also can provide a broader screen since the range of growth
5 conditions can be reflected differently in changes in a range of phenotypic sensors. An altered biomolecule, the presence of a modulator, or a combination of the two produce a range of cellular effects resulting in characteristic effects on a phenotypic sensor, such as the
10 growth of the cell, in different culture conditions. As a particular example, the ability of a cell to use specific substrates to support growth is altered by stress conditions, including altered biomolecules in the cell and the presence of a modulator. Thus, the presence of a
15 modulator acting on a particular biomolecule can cause a wild type cell to exhibit the same growth pattern as a cell having an altered version of that biomolecule. Likewise, the presence of an unknown modulator can cause a wild type cell to exhibit the same growth pattern as
20 that cell type exhibits in the presence of some previously known modulator. In particular embodiments, the plurality of growth conditions includes a variety of different growth media which differ in the carbon source available to support growth. Comparison of the patterns of
25 substrate utilization is then indicative of the cellular target of the modulator.

"Growth conditions" refers to an external cellular environment which is appropriate for growth of at least some cell types. Thus, the conditions include an
30 appropriate physical environment as well as an appropriate energy source. In general, in the methods of this

invention the growth conditions are selected to be appropriate for growth of a wild type cell.

As indicated above, in a preferred embodiment, the method further includes comparing the growth of the
5 cells in a plurality of different media which differ in their carbon source. (The ability to grow in particular media is the phenotypic sensor in this case.) It was determined that the ability of the cell type having an altered biomolecule, to utilize various carbon sources for
10 growth, can be mimicked by agents or modulators that act upon that biomolecule or its related pathways. That is, in cells having altered biomolecules, those cells are under stress and may have a reduced central function. This stress on the cell alters its ability to use one or
15 more carbon sources in a manner which reflects the mechanism of utilization of those carbon sources. Such a method provides a phenotypic profile (in this case a metabolic profile) of the activity of modulators of the biomolecules and allows analysis of the mechanism of
20 action of the modulators based on the carbon utilization of the cell. Examples of such analyses are provided in the Detailed Description below.

The term "carbon source" refers to a carbon-containing compound present in a growth medium which can
25 be utilized by at least some cell types as an energy source to support growth and/or as a supply of carbon for synthesizing needed molecules. The ability of a particular cell type or microbial strain to utilize a particular compound thus is a characterizing parameter for
30 a cell type. Examples of such carbon sources include, for example, a variety of simple sugars.

As noted above, it is preferable to utilize multiple comparisons, therefore, for the above methods, and generally for the methods of this invention, it is preferable to utilize a plurality of cell types. In particular, it is preferable to utilize a plurality of different cell types having different altered biomolecules.

It is also preferable in the above methods and in other methods of this invention, to examine a plurality of phenotypic sensors to determine the presence and/or effects of a modulator. This provides both a broader range of information concerning the functioning of the modulator, as well as a broader screen (due to screening a broader range of targets and mechanisms).

In a preferred embodiment, the conditional growth phenotype of a cell type is a temperature sensitive (ts) phenotype, usually a heat sensitive phenotype. Thus, such a cell type would preferably be grown at a semi-permissive temperature, i.e., a temperature intermediate between the permissive and restrictive temperatures for that cell. Preferably, but not necessarily, such a temperature sensitive phenotype is conditionally lethal, meaning that the cells die at the restrictive temperature. While temperature sensitive phenotypes (both heat and cold sensitive) are preferred in the current embodiments, other conditional phenotype cells can be isolated, and may be preferable in particular applications. Such other phenotypes include, but are not limited to sensitivity to pH or osmotic pressure.

By "microbe" is meant to encompass generally simple, microscopic organisms. In most cases these are

haploid organisms such as bacteria, fungi, and viruses, for example, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Candida albicans*, *Candida glabrata*, *Escherichia coli*, *Staphylococcus aureus*, and the like. (Fungi include
5 yeasts.) Haploid organisms, generally having only one form of any gene, are thus more readily manipulated by genetic means. However, diploid organisms are also useful in the methods of this invention, both in screening for agents active against those organisms, and also as models
10 of the effects of an agent in mammalian (e.g., human) cells.

In a related aspect, the invention provides a method of screening for a modulator of a biomolecule by simultaneously growing a plurality of cell types having
15 altered biomolecules in the presence of a potential modulator, and determining whether the growth of any of the cell types is altered in the presence of the potential modulator. The plurality of cell types are preferably separately in contact with the potential modulator.
20 Further, the plurality of cell types can be grown in the presence of a number of different potential modulators at the same time. The cell types having altered biomolecules may, but do not necessarily have conditional phenotypes. Preferred embodiments of this aspect are similar to those
25 for the first aspect.

Also in preferred embodiments, the method further comprises simultaneously determining the mechanism of action of a modulator by comparing the effects of the modulator on one or more phenotypic sensors, such as the
30 pattern of growth of the plurality of cell types, in the presence of the modulator to the pattern(s) observed when

the cell are grown in the presence of a modulator having a known mechanism of action. Further, the method comprises simultaneously determining the mechanism of action of a modulator by comparing the pattern of utilization of a variety of carbon sources or other phenotypic sensors by the normal cell in the presence of a modulator to that of a cell type of known mutation in the absence of a modulator or to that of a normal cell in the presence of a modulator of known mechanism.

10 The term "simultaneously" in the context of the methods of this invention refers to at least partially concurrent operation of some function. Thus, for simultaneous growth of a number of different cell types, the different cell types are all grown at the same time for at least a part of the entire period of, preferably however, all the cell types involved in a single operation of one of the methods of this invention are grown fully concurrently, that is, culturing is begun and ended at the same time for all cell types.

20 In reference to the operation of a modulator on a biomolecule, the term "mechanism of action" refers generally to the way in which a modulator exerts its biological effects on a cell. Generally, this refers to a specificity at least to the level of a gross cellular function, for example, protein metabolism, nucleic acid metabolism, or cell wall biosynthesis. Thus, in the context of this invention, a mechanism of action is "known" if the knowledge of that mechanism is available to the operator of one of these methods prior to examining the results of the method. This does not mean that the

operator has actually obtained that knowledge, but only that is available to that person.

In distinction from "mechanism of action", a "cellular target" or "genetic target" refers to a greater degree of specificity. These terms refer to a cellular biochemical pathway which involves or significantly affects the function of a particular biomolecule. Thus, the term may refer to a particular biomolecule which is directly affected by a modulator or to the biochemical pathway which significantly involves a particular biomolecule.

As used in reference to the methods of this invention, the term "pattern of growth" refers to the accumulated results of differential growth test over a number of different cell types and/or over a number of different growth conditions. Similarly, the term "pattern of carbon source utilization" refers to the accumulation of substrate utilization tests over a number of different cell types and/or over a variety of different growth media having different carbon sources.

Also in particular embodiments of the above aspects, at least one alteration of a biomolecule lowers the activity of that biomolecule.

Referring to the biological function of a particular biomolecule, the "activity" of that biomolecule refers to the level of biological function exhibited by the total pool of a particular biomolecule in a cell or group of cells of a particular type. Thus, in general, the term refers to the level of biological function of a specified amount of a biomolecule but can also refer to the total level of function of that biomolecule in a cell,

thus, the latter situation can include cases where a particular biomolecule is underexpressed, i.e., where a smaller quantity of the molecule is produced in the cell than in a normal cell of that general type.

5 In yet other particular embodiments of the above aspects, cell growth is measured by an indirect method, or an altered biomolecule is overexpressed, or an altered biomolecule is expressed at a lower level in a particular cell type than the wild type analog in a wild type cell
10 line or strain.

The terms "overexpressed" and "normal level" and "lower level" or "underexpressed" are distinguished by reference to the numbers of active molecules of that type present in a cell. Overexpressed refers to a
15 significantly higher level of expression (20% or greater), while lower level refers to a significantly lower level (20% less or lower). Thus, normal level refers to the numbers of active molecules which are present in a wild type or reference cell of a particular general type. One
20 approach for obtaining such altered expression levels is by the cloning of a regulator sequence which is transcriptionally-linked with the gene for the altered biomolecule, which results in a change in the level of transcription and/or translation of that gene.

25 In two related aspects, this invention provides methods for screening for or characterizing a modulator of a biomolecule. The methods involve simultaneously performing a set of assays to characterize multiple properties of a modulator at the same time. This is a
30 departure from the usual practice of examining a single property of a compound at a time; instead it focuses on

providing a range of information on a compound's properties. This range of information, available at the initial stage, at or just following identification of a modulator, enables better prioritization of candidate compounds, such as compounds for therapeutic uses. Typically, though not necessarily, this multiple assay (multi-channel) approach is performed using 96-well microtiter plates. Also typically, though not necessarily, the results of the multiple assays provides a pattern of results which can be compared to results for a modulator(s) for which certain results or characteristics are known.

This multichannel method is particularly suitable for inclusion of screening and characterizing methods described above, which are based on determining the effects of a modulator on one or more cell types having a conditional growth phenotype. Therefore, in preferred embodiments, the method includes determining the effects of the modulator on a cell type(s) having conditional growth phenotype(s). Also in a preferred embodiment, the method includes an assay(s) which allows determination of the cellular target of the modulator. Typically, such a target determination will involve identification of the pattern of effects of the modulator on a number of cell types.

The "assay" refers to a test the results of which provide information on a value or characteristic associated with the thing being tested or assayed. Thus, for example, different assays may examine the numbers of cells present, whether a particular carbon source that is utilized, the amount of active compound in a particular

solution or suspension as well as broad variety of other values and properties.

A "property" of a modulator can refer to both physical and functional properties. Thus, the term can
5 refer to characteristics such as stability of a compound in a particular solution, the effects of a compound on the growth of one or more cell types, the effects of a particular compound on the growth substrates utilized by a particular cell type, the serum binding of a particular
10 compound, among others.

The term "characterizes" in reference to the properties of a modulator refers to at least a partial determination of a property or properties of the compound. The property being characterized may but need not be
15 directly determined by the result of a particular assay characterizing a particular property or may involve drawing inferences from a number of different assays or observations.

A "method for evaluating" a modulator refers to
20 the determination of the results of a variety of tests involving that modulator. In general, such evaluating characterizes the modulator with respect to a number of different properties and provides a body of information which at least partially distinguishes that compound from
25 other compounds.

In further preferred embodiments, at least one assay is useful to determine one of the following properties of a modulator: serum inactivation, stability in a solution or suspension, the frequency or mechanism of
30 development of resistance to the modulator, the solubility of the modulator in a solvent or solution, the cellular

toxicity of the modulator, and the breadth of spectrum of the modulator.

As noted, assays useful in this invention examine a variety of different properties of modulators. In this context, the term "stability" refers to the resistance of a molecule to structural changes in a particular environment. Such structural changes may involve cleavage rearrangement or other chemical modifications.

The term "serum inactivation" refers to the loss of biological activity of a particular compound in a particular mammalian serum. Such inactivation generally involves some type of structural modification, often cleavage; it may also include simple inactivation by binding of a particular compound to serum proteins.

The terms "a solvent", "solution", and "suspension" have their usual chemical meaning. Thus, solvent is the major component in a molecular mixture of two or more components. A solution is a molecular mixture of one or more components with respect to another component. A suspension is a physical dispersion of one component in another component in the context of the methods of this invention. A solvent is a liquid and the solutions and suspensions are with reference to the major liquid component. Thus, the term "solubility" refers to the amount of a material (a solute) which will go into solution in a particular solvent. In general, in the methods of this invention, the relevant solvents are aqueous solvents such as water, physiological saline, buffers, media or serum.

With respect to the "development of resistance" of a cell for a particular modulator, the term refers to

an increase in the amount of the modulator needed to elicit a particular level of biological effect from a particular cell. Such development of resistance commonly occurs at different frequencies depending on the mechanism
5 of action of a particular modulator and for different cell types. Thus, the frequency of development of resistance refers to how often cells having such a change in their response to a modulator appear in a particular cell population.

10 With respect to yet another property of modulators, the "cellular toxicity" refers to the lethal effect of a compound for a variety of cells. Such toxicity is typically determined with respect to a particular reference cell; for therapeutics the reference
15 cell is generally a mammalian cell. Such cellular toxicity in general results from the effect of a compound on a component or class of components which are present in most relevant cells of interest.

In reference to the biological activity of a
20 modulator, the term "breadth of spectrum" refers to the range of cell types for which the modulator exhibits a particular biological activity. Thus, for example, for a modulator which is an antibacterial agent, the relevant breadth of spectrum typically refers to the range of
25 bacterial strains and species for which that antibacterial agent has its antibacterial activity.

In a particularly preferred embodiment, the methods further comprise determining a molecular toxicity profile of a modulator, by comparing the pattern of
30 toxicological results of the modulator in the plurality of cell types with the effects of another, or preferably a

plurality, of other compounds. Since the mechanisms and molecular interactions of such other compounds provides comparative information on those properties for the test compound, preferably this method includes characterizing the molecular interactions of the test modulator in a cellular context. This provides predictive capability of the behavior and biological effects of that compound in a biological application. In particular embodiments, it is useful to analyze the molecular toxicity and cellular interactions of compounds by determining the effects of those compounds in an array of bacterial and/or fungal strains (e.g., *Sacchromyces* strains), as well as mammalian cells. Notably, the *Salmonella typhimurium* and *Staphylococcus aureus* ts mutant strains described herein, and other similar strains, provide a suitable cell type panel for such analysis.

In another aspect, the invention features a method for determining a phenotypic profile of one or more cell types by determining the effects of a modulator or an altered biomolecule on a plurality of different phenotypic sensors, preferably in a plurality of different simultaneous assays. Preferably the plurality of different assays characterize a number of different properties of the cell types. The plurality of different assays can, for example, include evaluation of a phenotypic sensor which differs between wild type and mutant cells in the presence/absence of a modulator, such as tests for growth in a number of different growth conditions. Such growth test include growth in different growth media having different carbon sources.

As used herein, the term "phenotypic profile" refers to a pattern of results identified for a particular cell type over a number of different tests or assays. Therefore, as indicated above, one type of phenotypic profile can be established for a cell type based on the pattern of carbon source utilization/non-utilization observed in a number of different carbon source utilization growth tests (preferably performed simultaneously). A phenotypic profile can also include other indicators of metabolic function, either additively or alternatively. These include, for example, examining RNA transcripts whose expression is sensitive to changes in the cell's physiology, determining the intracellular pool levels of important cellular metabolites such as ribo- and deoxyribonucleotides, cAMP, alarmones, acyl-phosphate, and polyphosphates, determining the production of particular extracellular products such as particular secondary metabolites, mixed acids from fermentation, and quorum-sensing peptides, and by the use of display methods such as 2-dimensional gel electrophoresis of cellular proteins (2-D gels).

In a further aspect, the invention provides a method for characterizing the mechanism of action of a modulator of a biomolecule. The method involves determining the effects of a modulator on a phenotypic sensor, such as the pattern of growth, in a number of cell types which each have different altered biomolecules. The phenotypic sensor effects for the different cell types is compared to the effects of a known modulator. Such comparison indicates whether the mechanism of action of the test modulator is similar to the mechanism of action

of the known modulator. If the mechanism of action of the known modulator is known, the mechanism of action of the test modulator is thus narrowed to include this mechanism and closely related mechanisms. Characterization of the mode of action may include identification of the cellular target on which the modulator acts. This method thus provides a rapid approach to identifying a mechanism of action by comparison to previously identified mechanisms of known modulators. In particular, this provides early characterization of a compound having antimicrobial activity, without the need for structural identification of the compound or complex mechanism studies. This approach is especially useful when a large variety of different cell types are utilized which differ significantly in their susceptibility to different antimicrobial agents, e.g., differential susceptibility of various bacterial strains to β -lactams, aminoglycosides, glycopeptides, and tetracyclines.

Similarly to the above aspects, in preferred embodiments, at least one of the cell types is a conditional growth mutant, meaning it has a conditional growth phenotype, such as a temperature sensitive phenotype, and is preferably grown under semi-permissive conditions. Also in a preferred embodiment, the characterizing comprises identifying the cellular target of the modulator.

In a related aspect, this invention provides a method for characterizing the mechanism of action of a modulator of a biomolecule by comparing the effects of the modulator on a phenotypic sensor in a cell type having a particular normal biomolecule with the effects on that

phenotypic sensor of particular altered biomolecules in other cell types. As indicated in aspects above, the effects of a modulator which targets a particular normal biomolecule can mimic the effects of an alteration of that biomolecule which changes its function. Therefore, such comparisons indicate whether the mechanism of action of the modulator involves a biomolecule corresponding to any of the altered biomolecules in the other cell types. Typically, the normal cell type would be a parent strain or cell line, and the cell types having altered biomolecules would be mutant derivatives of that parent cell type (or closely related cell types).

Preferred embodiments of this aspect are similar to those indicated for the above aspects, including utilizing a plurality of phenotypic sensors, or cell types having conditional growth phenotypes, or cell types from particular organisms, such as bacteria, fungi, and mammalian cancer cells.

In two related aspects, the invention provides methods for characterizing natural products preparations. The first involves comparing the effects of a test natural products preparation on a phenotypic sensor in a plurality of cell types having altered biomolecules with the effects of a second natural products preparation or a known modulator. Comparison of the patterns of effects for the plurality of cell types in the presence of the different preparations (or the preparation and the known modulator) provides an indication of the similarity or difference of a modulator(s) present in the preparation being tested to those present in the reference natural products preparation or the known modulator. This approach

provides a rapid method for prioritizing natural products preparations for further development of useful modulator such as antibacterial agents, including the dereplication of natural products preparations having a desired activity. In addition, if the mechanism of action of the known modulator is known, the method can also provide an indication of the mechanism of action of a modulator in the natural products preparation if the growth pattern of the cell types is consistent with a similar mode of action, even if the two modulators are different.

The second related aspect involves comparing the effects on one or more phenotypic sensors of different altered biomolecules in a plurality of cell types to the effects of the natural products preparation on those phenotypic sensors in a cell type having normal biomolecules corresponding to those altered in the other cell types. Similar to aspects discussed above, the creation of phenotypic effects by the modulator which mimic the effects of a particular altered biomolecule is an indicator that the modulator acts on that biomolecule or a related biochemical pathway.

Preferred embodiments of these two aspects are similar to those indicated above for previous aspects.

The term "natural products preparation" refers to a compound or mixture of compounds, which can be a complex uncharacterized mixture, which is prepared from a living organism source such as plants, animals or microbes. Typically, such preparations are partially fractionated mixtures derived from a culture medium of a particular cell or directly from the cells of a particular type. However, the term can also refer to unfractionated cell

culture extracts or lysates or to fractions which have been highly enriched for one or more compounds.

Since this method is useful for dereplication of natural products preparations which show modulator
5 activity, in a preferred embodiment the characterizing comprises determining whether the first natural products preparation inhibits a different genetic target than the second preparation of the known modulator. This means that the method characterizes the target similarities or
10 differences of modulators in the two preparations, or in the preparation and the known modulator.

In particularly preferred embodiments of each of the above aspects, a cell type is a microbe, including bacteria selected from the group consisting of the
15 *Staphylococci*, the *Pseudomonads*, the *Enterococci*, the *Enterobacteriaceae*, and the *Streptococci*. Of particular interest are the common pathogenic species, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Enterococcus faecalis*, and
20 *Streptococcus pneumoniae*. Specific embodiments utilize *Staphylococcus aureus*. Also in preferred embodiments, a cell type is a fungus, such as *Saccharomyces cerevisiae*, *Aspergillus niger*, *Candida albicans*, and *Candida glabrata*. Likewise, in preferred embodiments of the above aspects,
25 a cell type is a mammalian cancer cell.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Figures

Fig. 1 shows the fold increase in sensitivity toward 12 antibacterial agents and a generally toxic agent for 3 temperature sensitive mutants of *Salmonella typhimurium*. These are mutants of DNA gyrase subunit A (*gyrA212*, *gyrA215*, and *gyrA216*, grown at a semi-permissive temperature (35°C)). Hypersensitivity to antibacterial agents acting on DNA gyrase, but not to other classes of drugs or toxic agents. The data demonstrate that growth conditional mutations in a known target cause hypersensitivity to target inhibitors.

Fig. 2 presents the hypersensitivity profiles of a set of temperature sensitive mutants of *Salmonella*, for a variety of antibacterial agents with characterized modes of action, compared to the sensitivity profile of wild type.

Fig. 3 illustrates a variety of types of interactions which exist between different essential genes, and which can create differential responses in screens using growth conditional mutants.

Fig. 4 illustrates a possible arrangement of a multichannel screen plate using conditional growth mutants with mutations affecting 5 different cellular processes plus controls.

Figs. 5-9 illustrate the carbon source utilization for three *ts gyr* mutants, with comparisons to mutants in other genes and to wild type in the presence of an antibiotic. Carbon source compound codes are as shown below:

Carbon source compound codes

	A6	Tween 80
	B7	M-inositol
	C2	β -methyl-D-glucoside
	C3	D-psicose
5	D4	Formic acid
	D10	α -hydroxybutyric acid
	E2	Itaconic acid
	E3	α -ketobutyric acid
	E8	Propionic acid
10	F2	Succinamic acid
	F4	Alaninamide
	H7	2-amino ethanol

Fig. 10 illustrates a multi-channel implementation in microwell plates in which multiple cell 15 types (e.g., growth conditional mutants) are used on each plate to create a phenotypic profile of a single compound. Multiple plates are used to screen different compounds. As illustrated, an implementation can characterize a number of properties of a compound to 20 allow the identification of desirable profiles (phenoprints) or targets.

Fig. 11 presents the structures of two compounds which exhibited the same inhibition profiles for a set of temperature sensitive *Staphylococcus aureus* mutants, 25 showing the structural similarity of the compounds.

Fig. 12 presents the fold increase in sensitivity of a set of *Staphylococcus aureus* temperature sensitive mutants for a variety of compounds which inhibit growth of *Staphylococcus aureus* wild type, but 30 which have uncharacterized targets of action.

Figs. 13 illustrates a possible multi-channel screening plate which is designed for the identification of compounds acting on DNA gyrase, showing hypothetical results (-) which could be obtained for a new 5 antibacterial agent targeting gyrase subunit A.

Fig. 14 illustrates the types of anticipated inhibition profiles of different growth conditional mutants for a variety of test compounds, indicating that the number of mutants affected by a particular compound 10 is expected to vary.

Fig. 15 shows the proportion of compounds (from a total of 65) which significantly inhibited the growth of varying numbers of temperature sensitive mutants in a screen of uncharacterized growth inhibitors of 15 *Staphylococcus aureus*

Fig. 16 shows the potency (MIC values) of a number of growth inhibitors which affected 0, 1 or more than 3 temperature sensitive mutants of *Staphylococcus aureus* in a screen of uncharacterized growth inhibitors.

20 Fig. 17 shows the number of hits for each of the temperature sensitive mutants of *Staphylococcus aureus* in a screen of 65 uncharacterized growth inhibitors.

Fig. 18 shows some advantages of a multichannel genetic potentiation screen using growth conditional 25 mutants over traditional biochemical screens with either a known target or an unknown cloned gene.

Fig. 19 illustrates a strategy for selecting dominant lethal mutants for use in screens for antibacterial agents, not requiring hypersensitivity.

Fig. 20 is a table describing a variety of multi-channel screening goals (methods) and appropriate approach for implementing such methods.

The present invention provides methods for identifying and evaluating modulators of biomolecules. Such modulators may either inhibit or enhance the function of a particular biomolecule, however, for many therapeutic uses, inhibitors are appropriate. Thus, this invention is useful, for example, in the identification and development of antimicrobial agents (including antibacterial agents and antifungal agents) and anti-cancer agents, as well as other therapeutic compounds.

This invention includes two related elements. Conditional growth mutant cells contain an altered biomolecule which is involved in an essential cellular function. Under permissive conditions, the biomolecule is sufficiently functional to allow normal or near normal growth of the cells. However, under restrictive conditions, the function of the altered biomolecule is crippled to an extent that the cells grow significantly slower or not at all. Under intermediate conditions, termed semi-permissive conditions, the function of the altered biomolecule is partially crippled. It is shown in the Examples below that conditional growth strains can be isolated such that the partially crippled biomolecule is hypersusceptible to inhibitors acting on that biomolecule or related biochemical pathways. Such strains also exhibit characteristic and identifiable phenotypic patterns under semi-permissive conditions in other characteristics such as carbon source utilization.

Therefore, the first element of this invention utilizes phenotypic sensors of a cellular alteration(s) (generally a defect) by evaluating the differences or similarities between wild type cells and mutant cells under appropriate conditions. Such phenotypic sensors reflect the metabolic alterations caused by a change in a particular biomolecule(s), especially an alteration in an essential biomolecule. Appropriate phenotypic sensors can be established by determining detectable phenotypic characteristics which differ between wild type cells and one or a number of different mutated derivatives of that wild type. (Generally, the characteristic will differ also between mutants having different altered biomolecules.) These phenotypic sensors reflect what a cell can do under particular conditions (e.g., grow; utilize a specific substrate), and/or the status of a particular cell component or molecule (e.g., metabolic pools; transcripts present; protein patterns).

Since the metabolic alterations result from specific altered biomolecules, a modulator which targets that biomolecule can cause the phenotype of a wild type cell to essentially reproduce the phenotype of the corresponding mutant cell type and/or can cause hypersusceptibility of the mutant as compared to the wild type. As suggested above, these effects can be monitored by examining a relevant phenotypic sensor(s). One such phenotypic sensor is the distinctive pattern of carbon source utilization under stress conditions, where to pattern is indicative of the particular altered biomolecule. Another important phenotypic sensor is based on the hypersusceptibility of conditional growth

mutant cells grown under semi-permissive conditions, such as temperature sensitive bacterial strains. Such strains can be used to detect the presence of a modulator(s) of a biomolecule and/or to characterize the mechanism of action of the modulator. As indicated in the Summary, conditional growth cell types are not limited to heat sensitive types, but include other cells whose growth is conditional on another culture parameter(s), such as cells hypersensitive to pH, osmotic pressure, and low temperature. This hypersensitive effect can be termed "genetic potentiation", as the altered biomolecule (genetic component) potentiates the effects of a modulator acting on that component. As noted above, such cell may exhibit a pattern of phenotypic effects in addition to hypersensitivity.

Thus, phenotypic sensors can be roughly grouped into at least two general categories: 1) those which are reflected as a greater effect of a modulator on a mutated cell type than on the wild type, 2) those for which a modulator causes a wild type cell to essentially reproduce the phenotype of a mutated cell with respect to the particular characteristic being monitored.

The second element of the present invention represents a new approach to identifying and characterizing modulators of biomolecules by incorporating a set of different assays together to provide a broader range of evaluation criteria at an early stage of development of a modulator such as an antibacterial agent. Such simultaneous acquisition of a range of information concerning a compound is particularly beneficial for prioritizing modulators for

further development as therapeutics. This approach, termed "multi-channel screening", includes the use of cells having conditional growth phenotypes as above, but also includes the use of other assays which do not depend on the use of such mutant cells. The selection of assays for a particular screen will usually vary depending on the specific application. Those assays can be of many different types, utilizing different phenotypic sensors (as discussed above), as well as other assays which may not involve mutant cell types.

The discussion and examples below emphasize screening and evaluating antibacterial agents, however, the present invention is also appropriate in identifying and evaluating other types of modulators, including anti-cancer agents.

Conditional Growth Cell Types - Hypersensitivity and TS Mutant Phenotypic Profiles

As indicated above, conditional growth cell types are not limited to heat sensitive cells, nor to bacterial strains. In particular, such cell types can be isolated in bacterial, fungal, and mammalian cells. The methods for isolating such cells are generally known to those skilled in the art, but typically involve isolating and testing mutants of some cell type of interest. The mutagenesis can occur by any of a variety of methods, including spontaneous mutagenesis, chemical mutagenesis, physical mutagenesis, site-directed mutagenesis, and *in vitro* mutagenesis. Mutants having a particular conditional growth phenotype obtained by any method are isolated, typically by selecting cell colonies which grow

under the relevant permissive conditions and not under the relevant restrictive conditions.

In addition to identifying new targets for drug discovery, the conditional growth mutants are useful for screening for modulators of the targets corresponding to the altered biomolecules, even before the genes or biochemical targets are fully characterized. The methodology can be whole-cell based, is more sensitive than traditional screens searching for strict growth inhibitors, can be tuned to provide high target specificity, and can be structured so that more biological information on test compounds is available early for evaluation and relative prioritization of hits.

Certain of the screening methods are based on the hypersensitivity of conditional growth mutants for compounds acting on an altered biomolecule related to the conditional growth phenotype. For example, conditionally lethal ts mutants having heat sensitive essential gene functions are partially defective at a semi-permissive temperature. As the growth temperature is raised, the mutated gene product causes a progressively crippled cellular function. It is the inherent phenotypic properties of such ts mutants that are exploited for modulator (e.g., inhibitor) screening.

Each temperature sensitive mutant has secondary phenotypes arising from the genetic and physiological effects of the defective cellular component. The genetic defect causes a partially functional protein that is more readily inhibited by drugs than the wild type protein. This specific hypersensitivity is exploited for screening purposes by establishing "genetic potentiation" screens.

In such screens, compounds are sought that cause growth inhibition of a mutant strain, but not of wild type. Such compounds are often inhibitors of the wild type strain at higher concentrations.

5 Also, the primary genetic defect can cause extended physiological changes in the mutant cells, even in semi-permissive conditions. As an example, a particular mutation can create the necessity for full function of biochemically related proteins upstream and
10 downstream of the primary target. Such effects cause concurrent hypersensitivity to agents that inhibit these related proteins, in addition to agents that inhibit the genetically defective cellular component. The effects of the physiological imbalance will occur through metabolic
15 interrelationships that can be referred to as the "metabolic web". Thus, in some cases, the initial genetic potentiation screen has the ability to identify inhibitors of either the primary target, or biochemically related targets.

20 Determination of hypersusceptibility profiles

General Methods for Examples 1 & 2

The minimal inhibitory concentrations (MICs) of various drugs and toxic agents were determined to evaluate the hypersusceptibility of *Salmonella*
25 *typhimurium* temperature-sensitive essential gene mutants.

The MICs were measured by using a standard micro broth dilution technique following the recommendations of the National Committee for Clinical Laboratory Standards (1994). Bacteria were first grown
30 in Mueller-Hinton broth at 30°C, diluted to 10^5 cfu/ml and

used to inoculate 96-microwell plates containing two-fold dilutions of antibiotics in Mueller-Hinton broth. Plates were incubated for 20h at a semi-permissive temperature (35°C) and the MIC was determined as the lowest dilution 5 of antibiotic preventing visible growth.

A two-fold difference in the susceptibility level of the mutant strain compared to that of the parental strain is within the limits of the experimental variation and thus a 3-4-fold decrease in MIC was 10 considered as a significant hypersusceptibility.

Example 1: Hypersensitivity of *gyr* mutants

The specific hypersensitivity of temperature sensitive mutations in a known target to inhibitors of that target is shown in Figure 1 with the susceptibility 15 profile of three *ts S. typhimurium* mutant alleles of the gyrase subunit A (*gyrA212*, *gyrA215* and *gyrA216*) grown at a semi-permissive temperature (35°C). The graph shows the fold-increases in susceptibility to various characterized antibacterial agents compared to that 20 observed with the wild-type parent strain. The data demonstrate the highly specific hypersusceptibility of these mutants to agents acting on DNA gyrase. Susceptibility to other classes of drug or toxic agents is not significantly different from the parent strain 25 (within 2-fold).

In addition, different mutant alleles show unique hypersensitivity profiles to gyrase inhibitors. One mutant shows hypersusceptibility to coumermycin ((*gyrA216*), one to coumermycin and norfloxacin (*gyrA215*), 30 and another to norfloxacin and ciprofloxacin (*gyrA212*).

Note that a mutation in the gyrase subunit A (*gyrA215*) can cause hypersensitivity to B-subunit inhibitors and could be used to identify such compounds in a screen. In addition, some *gyrA* mutant strains show no hypersensitivity to known inhibitors; potentially, these strains could be used to identify novel classes of gyrase inhibitors. Overall these results show that a selection of mutated alleles may be useful to identify new classes of compounds that affect gyrase function including structural subunit-to-subunit interactions. Thus, use of the properties of the crippled gyrase mutants in a screen provides a great advantage over biochemical-based screens which assay a single specific function of the target protein in vitro.

15 Example 2: Hypersensitivity profiles of
 Salmonella ts mutants

Demonstration of the generalized utility of hypersensitive screening with the conditional lethal mutants has been obtained (Figure 2) by collecting hypersensitivity profiles from partly characterized *Salmonella* conditional *ts* mutants. The table shows the increased susceptibility of the mutant strains to various characterized antibacterial agents compared to the wild-type parent strain. A two-fold difference in the susceptibility level is within the limits of the experimental variation and thus a 3-4-fold difference is significant.

A variety of hypersusceptibility profiles is observed among the *ts* mutants. These profiles are distinct from one another, yet mutants with related

defects share similar profiles. The *parF* mutants, which have mutations closely linked to the *Salmonella* topoisomerase IV gene, are hypersusceptible to gyrase subunit B inhibitors (black circle), although these 5 mutants are also susceptible to drugs affecting DNA or protein metabolism. Similarly, specificity within the hypersusceptibility profiles of two out of four *ts* mutants (SE7583, SE7587, SE5119 and SE5045) having possible defects in the cell wall biosynthesis machinery 10 are also observed (mutants *dapA* and *murCEFG*, black diamond). The latter mutants are also susceptible to other agents and share their hypersusceptibility profile with a mutant having a defect in the incorporation of radioactive thymidine (SE5091).

15 Thus, the hypersensitivity profiles actually represent recognizable interrelationships between cellular pathways, involving several types of interactions as illustrated in Figure 3. The patterns created by these profiles become signatures for targets 20 within the genetic/metabolic system being sensitized. This provides a powerful tool for characterizing targets, and ultimately for dereplication of screening hits. The hypersusceptibility profiles have been established for 120 *Salmonella* and 14 *Staphylococcus aureus* *ts* mutants 25 with a selection of 37 known drugs or toxic agents

Phenotypic Sensor Methodology - Mutant
Phenotypic Profiles

The conditional growth mutants are also used in phenotypic sensor methodology to provide a further 30 phenotypic profile, e.g., using carbon source utilization

profiles. With sufficient phenotypic sensors, a pattern or phenotypic profile of specific target inhibition is established. (A particular phenotypic sensor may also be useful alone, such as to identify a target by comparison between a wild type cell and one, or preferably a panel, of mutant cell types.) Therefore, mutant strains are evaluated to identify a diverse repertoire of phenotypes to provide this phenotypic profile. These evaluations include hypersensitivities to known toxic agents and inhibitors, carbon source utilization, and other markers designed to measure specific or general metabolic activities for establishing a mutant phenotypic profile that will aid in interpretation of modulator profiles.

As an example, in comparison to wild type strains or to ts mutants grown under permissive conditions, ts mutants fail to metabolize certain different carbon sources in semi-permissive growth conditions. The carbon sources not utilized by a specific mutant or group of mutants provide additional phenotypes associated with the crippled essential function. Moreover, some of these carbon source markers were also not used by the wild type strain exposed to sub-MIC concentrations of known drugs affecting the same specific cellular targets or pathways. For example, a sublethal concentration of the cell wall active agent, cefamandole, prevented the *Salmonella* wild type parent strain from metabolizing the same carbon source that was not used by either the *dapA* or the *murCEFG* cell wall related mutant. Thus, an inhibitor which targets a particular biomolecule essentially creates a carbon utilization phenotype in a wild type cell as is observed

for a mutant cell having that biomolecule altered (partially crippled).

As indicated in the Summary above, a phenotypic profile can incorporate other indicators of metabolic function, which can be included either alone or in combination. In addition to carbon source utilization, nitrogen source utilization can be examined. Likewise, RNA transcripts whose expression is sensitive to changes in the cell's physiology provide a monitor of cellular function. The intracellular pool levels of important cellular metabolites such as ribo- and deoxyribonucleotides, cAMP, alarmones, acyl-phosphate, and polyphosphates likewise serve as indicators of cellular function, as does the production of particular extracellular products such as particular secondary metabolites, mixed acids from fermentation, and quorum-sensing peptides. Further, analysis of display methods such as 2-dimensional gel electrophoresis of cellular proteins (2-D gels) provides an extensive analysis of cellular status. In general, any monitor which is capable of revealing differential cellular effects (differential phenotypic effects) is potentially useful for inclusion in a phenotypic profile.

In combination, interrelationships within and between essential and other cellular pathways are manifested in hypersensitivity and biosensor profiles that either singly, or preferably together, are employed for highly discriminatory recognition of targets and inhibitors. This information provides recognition of the target or pathway of compound action.

As was indicated above, analyses based on one or more phenotypic sensors typically involve either determining whether specific mutant cells exhibit a greater change in some sensor(s) in the presence of a particular modulator than wild type cells, or whether the presence of a specific modulator will cause wild type cells to essentially reproduce the phenotypes of one, or a characteristic set of mutant cell types. (Other analyses are also useful in certain applications.) In many situations, the actual comparison performed is between the observations of one, or preferably a plurality, of phenotypic sensor results for known modulators from a database of previously obtained results, and one or more experimental results of the effects of a putative modulator.

Example 3: Differential Carbon Source Utilization

The ability to metabolize particular substrates, such as carbon or nitrogen sources, has been used to characterize microorganisms (Lederberg, 1948, *J. Bacteriol.* 56:695) for genetic screening (Gutnick et al., 1969, *J. Bacteriol.* 100:215-219; Alper and Ames, 1975, *J. Bacteriol.* 121:259-266) and indeed to classify microorganisms for the purpose of taxonomy (Bochner, 1992, U. S. Patent No. 5,134,063). Indicators of metabolism can be growth, pH, or redox-sensitive dyes. Below, are described two applications in which the differential ability of a bacterial strain in the presence or absence of a test compound to utilize a particular substrate (or substrates) is exploited to

create a screen that identifies new antimicrobial agents. In one application, a wild-type strain, when grown in the presence of the test compound, will recreate a phenotype of a mutant test strain that has been previously characterized and shown to differ from that of the wild-type strain (with respect to substrate metabolism). In particular, the mutant strain is a ts strain whose phenotype is characterized under semi-permissive conditions. In the second application, a wild-type strain, when grown in the presence of the test compound, alters the profile of substrate utilization to recreate a previously characterized phenotype seen when the wild-type strain is exposed to a stressor (such as sub-MIC levels of antibiotic, sub-lethal levels of mutagens, etc.). Again, such a phenotype would have been previously characterized and shown to differ from that of wild-type.

Preliminary characterization of the utilization of large numbers of carbon sources can be carried out using manual or automated taxonomic screening devices, such as the Biolog MT, ES, GN or GP Microplate™. Alternatively, this can be performed using commercially available components. *Salmonella typhimurium* wild type and temperature-sensitive mutants, or wild type with sublethal levels of stressor were characterized. Briefly, strains were swabbed onto TSA plates and grown at a permissive temperature of 30°C for 6 hours to provide an inoculum. The inoculum (MacFarland standard -1 in 0.89% sterile saline solution) was prepared as per Biolog "Instructions for Use" handbook, with the exception that leucine was added to correct for mutants

housed in strains which were in a leucine-deficient background. Inoculum was added to Biolog GN or ES plates and incubated at the semipermissive temperature of 35°C overnight (~21hr). Carbon source utilization is read colorimetrically in a microplate reader at 600nm. Substrates that are not used similarly by wild-type and mutant are identified. Preliminary identification of substrates that are differentially utilized by wild-type and mutant, or wild-type +/- stressor, is followed by confirmation testing and optimization of the differential signal by varying concentration of substrate and other conditions (e.g., inoculum, temperature, media components). Typically, these steps were performed using Biolog MT plates or by using a redox indicator (such as tetrazolium violet, 0.0001%) in a minimal medium with a sole carbon source (substrate being tested), supplemented with a small amount of tryptone (0.06%) or proteose peptone (0.2%). It was possible to do this in 96-well microtiter plate format, which is adaptable to high throughput or multichannel screening.

Carbon source utilization differed significantly from wild-type in a majority of mutants and stressor conditions tested. Two particularly illustrative cases are that of the GyrA mutants versus wild-type, and that of wild-type +/- ciprofloxacin. First, as shown in Figures 5-9, three different gyraseA mutants differed from wild-type in their inability to use several carbon sources (the alphanumeric codes correspond to well locations on Biolog GN plates). This effect was significant in that a) the mutants resembled wild type in their ability or inability to use 70 out of 95 (70/95)

carbon sources tested, differing only in their utilization of a combined total of 15/95 carbon sources tested, and b) there was very significant overlap in the phenotypes with respect to carbon source utilization of the three gyraseA mutants. Mutants in other genes produced significantly different phenotypes. Second, the inclusion of sub-MIC levels of ciprofloxacin, whose molecular target in the cell is known to be gyraseA, reproduces the phenotype of one of the mutant alleles perfectly, and has significant overlap (46-71%) with the other two. Other classes of antibiotics administered at sub-MIC levels produced different phenotypes from the gyraseA/ciprofloxacin induced phenotype. Exceptions were cefamandole and chloramphenicol (40% overlap). Novobiocin, which targets gyraseB, produced a very similar phenotype to that of the gyraseA mutants and the ciprofloxacin induced phenotype.

While two different applications are described above, the two examples described herein bear a relationship to each other and allow further conclusions to be drawn. These examples reveal that a carbon utilization pattern provides a readout of the integrity of the function of that gene product or target. Thus, by screening as described, agents can be identified whose mode of action relates to the functional integrity of known gene products or known stressor targets.

Strain Validation and Screening Conditions

Hypersensitive strains (not conditional for growth) have been successfully used in the past for discovery of new drugs targeting specific cellular

pathways. (Kamogashira and Takegata, 1988, *J. Antibiotics* 41:803-806; Numata et al., 1986, *J. Antibiotics* 39:994-1000.) The specific hypersensitivities displayed by growth conditional cells, such as ts-conditional mutants, indicates that use of these mutants in whole cell screening provides a rapid method to develop target-specific screens for the identification of novel compounds. However, it is beneficial to eliminate mutants that will not be useful in semi-permissive growth conditions. Such mutant alleles may have nearly wild type function at the screening assay temperature. The simplest method for validating the use of ts mutants is to select those which show a reduced growth rate at the semi-restrictive growth temperature. A reduced growth rate indicates that the essential gene function is partially defective. More specific methods of characterizing the partial defect of a mutant strain are available by biochemical or physiological assays.

20 Multi-Channel Screening

The multi-channel screening aspect of this invention represents a change in the approach to identifying and characterizing useful modulators of biomolecules. Multi-channel screens simultaneously perform a large variety of assays, such as genetic potentiation assays, on a single compound to generate a compound profile. Such a compound profile can, for example, include information on target specificity through the inclusion of a large number of conditional growth cell types in the multi-channel screen. In

addition, further assays useful for the characterization and prioritization of compounds can also be included in a particular multi-channel screen, providing a much broader compound profile.

5 For multi-channel screens which include multiple genetic potentiation assays, a screen includes a panel of different conditional growth cell types, such as conditional growth bacterial mutant strains, which have different altered biomolecules. Depending on the
10 information desired from the screen, different types of cell panels can be selected (either separately or in combination) in which the different cell types are distinguished in various ways. For example, cell panels can be selected in which each cell type has a different
15 altered biomolecule. Such a panel provides broad information on target identification. In another example, each cell type in a panel can have a phenotypically distinguishable alteration in the same biomolecule (such as mutations at different residues in a
20 protein enzyme). This class of panel provides more specific distinctions of the action of a modulator on a known target. In yet another example of a type of cell panel, each cell type can have an altered component (i.e., an altered biomolecule) of a particular cellular
25 reaction pathway. Other selections can also be made, including combinations of different types of cell panels.

The cells of a particular panel(s) are, in general, tested with a variety of known compounds to provide a comparison database of the phenotypic effects
30 of those compounds. (Such phenotypic effects can be of many different types as discussed above.) Of particular

interest for inclusion in such comparison databases are the effects of therapeutic agent such as antibacterial agents and antifungal agents, toxic agents, and previously obtained natural products preparations. With 5 such a database available, the cell panel(s) can be used to screen compounds or preparations with unknown activity, or to characterize or evaluate compounds or preparations which are known to have some biological activity of interest. Comparison of the phenotypic 10 profiles of the test compounds to the profiles of known compounds provides broad, rapid information on a particular compound. Such information can be used in various ways, such as for the discovery of antibacterial or antifungal compounds active against essential genes 15 from the particular organism, for identifying cytotoxic compounds, and for identifying the mechanism of action of compounds which are known to have some interesting cellular effect. One benefit of such information is thus to allow early prioritization of the different active 20 compounds identified.

Creation of comparison databases can usefully involve characterizing the interactions for a large variety of compounds having some toxicity, which can for example be derived from various compound libraries. The 25 organisms utilized for such analysis can be derived from a variety of organisms. In particular, bacterial, fungal, and mammalian cells can be used. Of particular note, for these purposes are yeasts such as *Sacchromyces cerevisiae* and the bacteria *Salmonella tympthimurium* and 30 *Streptococcus aureus*. The analysis characterizes different types of molecular interactions, and thus the

results of the analysis are relevant between the different organisms.

Further, the multi-channel screen provides the ability to analyze the molecular toxicology characteristics of a modulator, both to determine the toxicity of the compound and to provide extensive information on the specific cellular interactions in which the compound engages, which then provides predictive ability of the behavior of a compound in a particular organism. In general, this application of the multi-channel invention involves determining a profile of the effects of a specific modulator in a number of different cell types. This profile is then compared to the profiles of the effects of other compounds to provide the information on the toxicity and cellular interactions. This is particularly significant for the development of agents intended for use in eukaryotic contexts, (e.g., mammalian cells). The information on the mechanism of action and cellular interactions (especially specific molecular interactions) of such other compounds provides comparative information on the characteristics of the test compound. Therefore, for this purpose, a database of the effects of toxic agents in eukaryotic microbes, such as yeast, serves as a convenient model of the effects of such compounds in mammalian cells. Such a database (and the profile of a test compound) will typically utilize a large number of cell types, which can include both different species and different strains (e.g., mutants) within a single species. The importance and usefulness of such a database increases as the number of compounds and cell

types increases due to the increasing number of comparisons which are then provided.

Thus, the usual use of conditional growth cell types in multi-channel screening can be summarized in the following way:

- a) Create an altered biomolecule in a cell by genetically altering the gene (and gene product) or obtain cells having such alterations;
- b) Form a panel of cell types having different altered biomolecules;
- c) Expose the panel of cell types to known modulators and determine phenotypic effects (phenotypic sensors) to create a comparison database;
- d) Expose the panel of cell types to test compounds or preparations and determine phenotypic effects, creating a phenotypic profile of the test material;
- e) Compare the phenotypic profiles of the test materials with those of the known modulators. Use statistical comparisons and pattern analysis as needed to compare the phenotypic profiles.

Figure 20 lists a variety of different types of uses of multi-channel screening and approaches and discriminators appropriate for each of those uses. However, it should be recognized that that list is not comprehensive, those skilled in the art will recognize many other suitable uses.

As noted above, both the phenotypic sensor (e.g., genetic potentiation) and multi-channel elements

of this invention are applicable to cells from a range of sources. Of particular interest due to the need for the development of therapeutic agents are bacteria cells, fungal cells, and mammalian cells (including mammalian 5 cancer cells).

A multi-channel screen can beneficially be run in a format such as 96-well plates. The use of the 96-well multi-channel screen format allows up to 96 different assays on each plate to characterize a single 10 compound. If additional assays are desired, one or more additional plates can be used at the same time. As shown in Figure 10, this format provides an immediate characterization or profile of a single compound. The more traditional arrangement, using up to 96 different 15 compounds per plate and a single assay, can also be readily accommodated by the genetic potentiation assays described above.

In comparing the multi-channel and the traditional screening arrangements, the multi-channel 20 screen format is generally compound-focused; prioritization of compounds run through the screen will occur, as decisions are made about which compounds to screen first. Each plate provides an immediate profile of a compound. The more traditional format is 25 target-focused: prioritization of targets will occur, as decisions are made about the order of targets or genetic potentiation screens to implement.

Multi-Channel Screen Advantages

A significant advantage of the multi-channel 30 screening approach is the ability to acquire a broad range of data with both clinical and product development

relevance at one time. This provides a new strategy, both for screening and for evaluating compounds and natural product preparations, which allows concurrent assessment of compounds in a variety of relevant assays rather than the currently employed pharmaceutical strategy of employing a series of testing and evaluating assays. Thus, this multi-channel approach provides a broad assessment of compound attributes, as well as the ability to identify a wider range of types of compounds, leading to greater efficiency and decreased cost in the process of identifying and developing bioactive compounds.

This is depicted, in part, by the *S. aureus* example shown below, where multi-channel screen design rapidly leads to the identification of compounds having interesting activity (hits) and provides some, or even all, of the necessary specificity information to prioritize compounds for further evaluation. Figure 12 illustrates the advantages of a genetic potentiation approach as the basis of a multi-channel screen design.

Overall, an approach using whole-cell phenotypic sensor (e.g., genetic potentiation) analysis of conditional growth (e.g., *ts*) mutants includes the selectivity of the biochemical screens (it is target-specific, or at least pathway-specific) and it is more sensitive than traditional screens looking for growth inhibitors due to the hypersensitive nature of the mutants. The phenotypic sensor approach also provides a rapid gene-to-screen technology and identifies hits even before the genes or biochemical targets are fully characterized.

Multi-Channel Screening Plate

The phenotypic profile results above, demonstrate that ts mutants show specific hypersusceptibility profiles in semi-permissive growth conditions. As a screening tool, the mutant inhibition 5 profile characterizes the effects of test compounds on specific bacterial pathways. Because the mutants are more sensitive than wild type strains, even compounds with weak inhibition activity can be identified.

An example of a multi-channel screen for 10 inhibitors of essential genes is shown in Figure 4. In this screen design, one plate serves to evaluate one compound. Each well provides a separate whole-mutant cell assay (i.e., there are many targets per screening plate). The included assays are genetic potentiation 15 assays, that is, conditional growth cells (e.g., ts-hypersensitive mutants) reveal compounds that are growth inhibitors at concentrations that do not inhibit the growth of the wildtype strain. The profile of mutant inhibition provides information on the compound's target 20 of inhibition. The ts mutants are grouped by their hypersensitivity profiles to known drugs or by their related defective genes. The figure illustrates the hypothetical growth inhibition results (indicated by "-") that would be obtained with a new antibacterial agent 25 targeting DNA/RNA metabolism.

A large variety of different multi-channel screen designs can be provided to fit specific needs or purposes. The choice of a broadly-designed screen (such as in Figure 4), or one focused on specific cellular 30 pathways, or even specific targets can be made by the appropriate choice of mutants. More specific screen

plates can use mutants of a specific gene target like DNA gyrase, or mutants in a specific pathway, such as the cell division pathway.

In addition to genetic potentiation assays, a variety of other assay types can usefully be included in multi-channel screening methods. One example is antibiotic potentiation assays. In one type of antibiotic potentiation, a sub-minimal inhibitory concentration of an inhibitor (e.g., an antibacterial agent) to which a conditional growth mutant is hypersensitive, the wild type cell type is tested to determine whether a potential modulator exhibits a synergistic effect with the known inhibitor. The observation of such a synergistic effect serves as evidence or corroboration of the mechanism of action of the modulator.

As an additional example, carbon source utilization assays as discussed above can be included in a multi-channel assay array to further characterize the effects of a modulator.

Figure 13 illustrates an implementation of a multi-channel screening plate designed for the identification of antibacterial agents directed against DNA gyrase, utilizing growth conditional (ts) mutants. Similar multi-channel screens can be designed using ts mutants to identify agents targeting other cellular functions such as protein metabolism, or agents affecting DNA metabolism but not related to DNA gyrase.

Example 4 - Evaluation of known growth inhibitors

In addition to testing known inhibitors of cellular pathways as described in the Examples above, uncharacterized growth inhibitors identified in other screens were also evaluated using temperature sensitive mutants. These growth inhibitors had uncharacterized targets of action. These compounds were previously shown to cause some growth inhibition of a standard laboratory strain of *Staphylococcus aureus* (strain 8325-4) at 5 mg/ml. The compounds were subsequently tested using a range of concentrations against a collection of *S. aureus* ts mutants (all derived from *S. aureus* 8325-4), to determine the MIC values, relative to wild type. Figure 12 summarizes the data generated using 52 *S. aureus* ts mutants and 65 growth inhibitor compounds (47 compounds not shown). The table reports the fold-increase in susceptibility of the ts mutants compared with the wild-type parent strain; values within two-fold of wildtype have been left blank in the table for ease of identifying the significant hypersensitive values.

The effects of the 65 test compounds on the ts mutants were mostly selective: for most compounds, a limited number of mutants were hypersensitive. Approximately one-third of all compounds showed identical inhibition of mutant and wild type strains (i.e., no mutants were hypersensitive to these compounds). Two compounds in Figure 12 showed strong inhibitory effects on about 50% of the mutants tested (compounds 00-2002 and 00-0167). Two additional compounds showed identical inhibition profiles (compounds 30-0014 and 20-0348, Figure 12). A preliminary analysis of these profiles is provided below.

The genetic basis of the hypersensitivity has been substantiated by two criteria. First, one compound (10-0797) strongly inhibited two mutants (NT52 and NT69) that both affect the same gene. Second, complementation of the temperature sensitive phenotype of these mutants resulted in loss of hypersensitivity.

Furthermore, the two compounds that had identical inhibition profiles (30-0014 and 20-0348) have very similar structures (Figure 11). Thus, the hypersensitivity profile provides a pattern that allows recognition of compounds with similar targets of action, even when the target may be poorly defined. The strong similarity in the structures of these compounds makes their common target of action likely. Based on the 15 mutants that were inhibited (*sea*, *dang*, and 3 uncharacterized mutants) the target of action of these compounds is not yet defined.

It is preferable to perform a screen of the uncharacterized inhibitors against a larger number of ts mutants. This screen employs preset compound concentrations and obtains the mutant inhibition profile for each compound. Computing the difference in the relative growth of parent and mutant strains in the presence of compounds provides a compound profile similar to that obtained by the MIC determinations of the first screen above.

A wide range of test compounds can be screened. Test compounds that are inhibitory for the wild type parent strain at the pre-selected concentration in the first screening run are retested at a lower concentration to generate an inhibition profile. Data analysis from

the screens described above showed that a significant growth reduction of mutant strains compared to the parent strain in the presence of the test compounds is a reasonable indicator of selective compound activity.

5 Further, compounds for testing can include compounds that show no growth inhibition of the wild type strain. The hypersensitivity of the mutant strains provides the ability to identify compounds that target an essential cellular function, but which lack sufficient
10 potency to inhibit the growth of the wild type strain. Such compounds are modified using medicinal chemistry to produce analogs with increased potency.

Analysis of hypersensitivity data

The grid shown in Figure 8 represents different
15 types of mutant inhibition profiles anticipated from screening of growth inhibitors, where "x" denotes inhibition of a particular mutant by a particular compound at concentrations much lower than for wildtype.

This grid shows compounds that cause growth
20 inhibition of more than one mutant (compounds A,C,D,E), compounds that inhibit just one mutant (compounds B,F) and one compound that inhibits no mutants (compound G). In addition, this profile identifies mutants inhibited by no compound (mutant 8), a single compound (mutants
25 1,6,7), and several compounds (mutants 2,3,4,5). In the preliminary screens described above, compounds were identified that fit some of these anticipated inhibition profiles.

In the preliminary screen, compounds that
30 inhibit the growth of the wild type strain were diluted to a point where growth inhibition of wild type no longer

occurred. In this situation, only mutants that are hypersensitive to a particular compound will fail to grow. Thus, even compounds considered "generally toxic" should show some specificity of action, when assayed with the hypersensitive mutant strains.

In the simplest interpretation, compounds that cause growth inhibition inhibit the function of one essential macromolecule. Some compounds may specifically inhibit more than one target macromolecule. However, since one of the targets will be most sensitive to inhibition, one target can be considered the primary target. Thus, a one-to-one correspondence between inhibitors and targets can be established. However, both the data, and less simplistic reasoning provide exceptions to the simple one-to-one relationship between targets and inhibitors. Further analysis and understanding of the complicating effects is necessary to make full use of the data. Some of the complicating effects are discussed below.

20 Compounds that affect many mutants. Certain compounds, such as detergents that target membrane integrity, or DNA intercalators, will have "general", rather than specific targets. These "general targets" are not the product of a single gene product, but rather 25 are created by the action of many gene products. Thus, in analyzing hypersensitivity profiles, compounds that affect many mutants may indicate action on a "general target". The profiles of known membrane active agents, and intercalators will provide information to recognize 30 uncharacterized compounds with similar effects.

Compounds that cause growth inhibition of more than one mutant may also arise when the affected mutants are metabolically related. These mutants may affect the same gene, or the same biochemical pathway. For example, 5 mutants defective in one of many cell wall biosynthetic steps may show hypersensitivity to compounds that inhibit any of these steps. Evidence for this type of effect was observed in the hypersensitivity patterns of known inhibitors (see Figure 2). This concept can be broadened 10 to include effects caused by the "metabolic web", in which far-reaching consequences may arise through characterized and uncharacterized interrelationships between gene products and their functions.

Overall, the hit rate was high when we 15 considered all compounds that were more active on mutants than on the parent strain. The histogram below (Figure 9) shows the hit rate for compounds that affected one, two, three, or more than three mutants in our prototype screen. The large number of compounds that affected more 20 than three different mutants was at least partly explained by the greater potency of this group of compounds. Figure 10 illustrates the potency of some of the hits found in the screen as evaluated by the MIC obtained for the parent strain *S. aureus* 8325-4.

25 In the prototype screen, compounds affecting more than 3 mutants were generally more potent but some may also be considered broadly toxic. The columns identified by an asterisk in Figure 10 represent 3 out of 4 compounds that were also shown to be inhibitors of 30 *Salmonella typhimurium* in another whole cell screen. Consequently, only the most hypersusceptible strain of a

group of mutants affected by the same compound should be considered as the primary target. Besides, the entire mutant inhibition profile of a specific compound is very useful and should be considered as its actual phenotypic profile in pattern recognition analysis.

Compounds that affect few (or no) mutants.

Since all compounds assayed in the preliminary screen inhibit the growth of the wild type strain to some degree (initial basis of pre-selection), such compounds indicate that the mutant population is not sufficiently varied to provide a strain with a corresponding hypersensitive target for each compound.

Mutants affected by many compounds.

Another complication of the simple one-to-one compound/target relationship will arise because of mutants that are inhibited by many different compounds. The relative number of compounds (% hits) that inhibited the growth of each mutant in the *S. aureus* pilot is shown in Figure 11. Several mutants were affected by many compounds. Several distinct causes of this are apparent. First, some mutants may have defects in the membrane/barrier that cause hyperpermeability to many different compounds. Such mutants will have higher intracellular concentrations of many compounds, which will inhibit metabolically unrelated targets. Other mutants may have defects that have far-reaching cellular consequences because their altered gene products sit at critical points in the metabolic web. Still other mutants may have specific alleles that are highly crippled at the assay temperature. For these mutants, the metabolic web

consequences are large because the specific allele has created a highly hypersensitive strain.

Mutants affected by few or no compounds. For the mutants that were hypersusceptible to fewer 5 compounds, it is possible that their mutations affect a limited metabolic web, that mutations provide a true specificity that was yet not revealed by any compound, or that these mutants have nearly full activity at the assay temperature. This analysis stresses the importance of 10 strain validation as indicated above.

In interpreting these patterns, the number of mutants screened and the total number of targets are also important variables. These numbers provide a simple probabilistic estimate of the fraction of the compounds 15 that should have a one-to-one correspondence with a mutant target in the sample that was screened.

Prioritization of Hits and Downstream Development

The early steps in a multi-channel genetic potentiation screen include the following:

- 20 • Pre-selection of mutant cell types (e.g., strains) for screening
- Pre-selection of desired test compounds based on structural features, biological activity, etc.
25 (optional)
- Testing of the chosen compounds at a pre-determined concentration (ex. 5 mg/ml)

- Analysis of inhibitory profiles of compounds against the mutant population and selection of interesting hits
- 5
- Confirmation of the selective inhibitory activity of the interesting hits against specific mutants
 - Secondary evaluation of prioritized hits.
- 10
- Genetic potentiation assays provide a rapid method to implement a large number of screens for inhibitors of a large number of targets. This screening format will test the capacity of rapid high-throughput screening. The capability to screen large numbers of 15 compounds will generate a large number of "hits" from this screening. Limitations in downstream development through medicinal chemistry, pharmacology and clinical development will necessitate the prioritization of the hits. When large numbers of hits are available, each 20 having reasonable *in vitro* activity, prioritization of hits can proceed based on different criteria. Some of the criteria for hit characterization can, for example, include:
- chemical novelty
- 25
- chemical complexity, modifiability
 - pharmacological profile

- toxicity profile
- target desirability, ubiquity, selectivity

Secondary tests will be required not only for the initial evaluation of hits, but also to support medicinal chemistry efforts. Many of these secondary tests can be included in multi-channel format along with the initial genetic potentiation assays. While the genetic potentiation tests are sufficient to identify and confirm hits, selection of hits for further development will necessitate establishment of the specific target of action. Using complementing clones to each of the conditional growth mutants, the gene corresponding to the altered biomolecule can be isolated. Such complementing clones can be isolated by techniques familiar to those skilled in the field, such as by construction and testing of clones from a genomic library. Equipped with the gene clones, selection of resistant alleles provides early evidence for the specific target. Subsequent efforts to establish a biochemical assay for rapid, specific and sensitive tests of derivative compounds will be aided by the over-expression and purification of the target protein, sequence analysis of the open reading frame (ORF) to provide early insight into novel target function, as well as a variety of physiological and biochemical tests comparing the mutant and wild type strain to confirm the novel target function, and aid in the establishment of biochemical assays for the targets.

Alternatives to Ts Hypersensitivity Screening
Appropriate for Multi-Channel Use

There are a number of additional strategies that can be undertaken to devise target-based whole cell screens. In order to implement these strategies, knowledge of the existence of the gene, the DNA sequence of the gene, the hypersensitivity phenotype profile, and the conditional mutant alleles will provide significant information and reagents. Alternative strategies include 10 those based on:

- over- and under-expression of the target gene
- dominant mutant alleles
- highly hypersensitive mutant alleles

15 Over- and Under-expression of Target Genes.

There are numerous examples of over-expression phenotypes that range from those caused by 2-fold increases in gene dosage (Anderson and Roth, 1977, *Ann. Rev. Microbiol.* 31:473-505; Stark and Wahl, 1984, *Ann. Rev. Biochem.* 20 53:447-491) to multi-fold increases in dosage which can be either chromosomal-encoded (Normark et al., 1977, *J. Bacteriol.* 132:912-922), or plasmid-encoded (Tokunaga et al., 1983, *J. Biol. Chem.* 258:12102-12105). The phenotypes observed can be analog resistance (positive 25 selection for multiple copies, negative selection for inhibition phenotype) or growth defects (negative selection for multiple copies, but positive selection for inhibition phenotype).

Over-expression can be achieved most readily by artificial promoter control. Such screens can be undertaken in *E. coli* where the breadth of controllable promoters is high. However, this method loses the advantage gained by whole cell screening, that of assurance that the compound enters the pathogen of interest. Establishing controllable promoters a cell type of interest is thus useful. For example, establishing a controllable promoter in *S. aureus* will provide a tool for screening not only in *S. aureus* but most likely in other Gram-positive organisms.

Dominant alleles. Dominant alleles can provide a source of screening capabilities. Dominant alleles in essential genes will prevent growth unless conditions are established in which the alleles are non-functional or non-expressed. Methods for controlled expression (primarily transcriptional control) provide the opportunity to identify dominant mutant alleles that prevent cell growth under conditions of gene product expression.

Equally useful will be mutant alleles that are dominant, but conditionally functional. A single mutation may provide both the dominant and conditional-growth phenotype. However, utilizing the existing collection of temperature sensitive alleles, mutagenesis with subsequent selection for a dominant allele may provide more mutational opportunities for obtaining the necessary dominant conditional alleles. There is precedent for such additive effects of mutations on the protein phenotype (T. Alber, 1989, *Ann. Rev. Biochem.* 58:765-798) as well as evidence to suggest that

heat-sensitive mutations, which generally affect internal residues (Hecht et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:2676-2680), will occur at different locations in the protein than dominant mutations, one type of which will affect protein-protein interactions, which are more likely on the protein surface.

The use of dominant conditional double mutants may have an additional advantage, since the hypersensitivity phenotypes may remain the same in the double mutant as in the single conditional mutant allele. In this case, a merodiploid carrying two copies of the target gene - one wild type, and one carrying the dominant conditional doubly mutant gene - would provide a sophisticated screening strain (see Figure 13). The screen would rely on the hypersensitivity of the dominant protein to inhibitor compounds. Under conditions of the dominant protein's function, cells will not grow, while inhibition of the dominant protein will allow cell growth. The temperature sensitive allele provides a basis for hypersensitivity of the dominant protein, relative to the wild type protein.

Hypersensitive mutant alleles - Additional mutants that display more pronounced hypersensitivities than the original conditional lethal mutants can be sought. Selection or screening procedures are based on the initial secondary phenotype profiles. These new highly hypersensitive alleles need not have a conditional growth defect other than that observed in the presence of the toxic agent or inhibitor. Such highly hypersensitive alleles provide strong target specificity, and high sensitivity to weak inhibitors. Such hypersensitive

alleles can readily be adapted for screens with natural products, and with synthetic or combinatorial libraries of compounds in traditional screen formats.

Multi-Channel Pattern Comparison and Data Analysis

5 The overall strategy for analysis of multichannel screen data is to: (1) group compounds with similar biological effects, (2) correlate the compound groups to specific targets, and (3) rank the compounds based on the additional criteria of pharmaceutical
10 desirability that have been designed in the multi-channel panel of assays.

The identification of similarities in compound profiles can be initiated by simple methods of inspection, however, graphical tools can simplify the
15 inspection process. Radial plottings of the compound profile provides a diagram of the data, which allows visualization of similarities between the profiles of individual compounds. Alternatively, multiple de Finetti diagrams can provide a method of visualization of
20 compound profiles.

As compound profiles are accumulated, statistical tools will become useful or even necessary to recognize appropriate grouping of compounds. Such statistical tools should be designed to provide
25 relationships between the compounds, based on the biological effects of the compounds.

Understanding of the relationships between genes will enable correct interpretation of the biologically meaningful relationships between compounds. Genes can be
30 grouped into clusters, based on current knowledge of

metabolic pathways. This clustering should reflect biologically and metabolically meaningful relationships between genes and gene products. A measure of the distance between genes in the relationship can be estimated. (i.e., This is more than just "related or "not-related"; more like a distance tree.)

Compounds can be grouped into clusters, based on the statistical analysis of their effect upon a panel of mutant strains (or a panel of assays). Such clustering should create relationships between compounds, based on their biological effects in the multi-channel panel. Multivariate statistical analysis, including identification of principal components and cluster analysis algorithms, provide the tools to perform this grouping of compounds.

Interpretation of the compound profiles requires creating a dictionary of mappings between the compound groups and the gene groups. These preliminary mappings should cause revision and updating of the appropriate cluster relationships and distances. Thus, the appropriate statistical analysis includes an learning and training phase of data interpretation, requiring more sophisticated algorithms, such as found in neural networks and other pattern-recognition algorithms.

Direct empirical mapping of compounds to specific genes can be accomplished by standard (though labor-intensive) methods used in the pharmaceutical industry for identifying a compound's mechanism of action. A database of information can be created of the performance of compounds with known mechanisms of action in the multichannel panel of assays. Such information

can provide constraints that limit the search space for mapping solutions, thus simplifying the computational problem, as well as reinforcing the correct solutions that are identified by computational methods.

5 The data analysis process provides the ability to determine similarities between compounds and natural products, based on the multichannel profile of assays. Likewise, the data analysis process provides the ability to determine similarities between the assays, based on
10 their performance with a number of compounds. This allows the ability to systematically refine and optimize the panel of assays to provide maximal information. When pairs of assays provide data that are completely correlated, the assays are redundant, thus one of the
15 assays can be discarded in favor of an assay that provides an independent measure for the compound profile.

Compounds and Preparations for Screening and Evaluation

As was suggested above in the Summary, a variety of different sources of modulators are appropriately
20 screened, characterized or evaluated with the methods of this invention. These include synthetic compounds (including compounds which are not structurally identified), natural products compounds or preparations, and combinatorial libraries. In use with such test
25 materials, these methods provide both initial screening information on a compound or preparation, and ,by comparing results for different modulators, information to dereplicate modulators. Thus, compounds or preparations can be identified which exhibit the same
30 phenotypic profiles and which therefore are expected to

have the same mechanism of action. This information is particularly useful in prioritizing natural products preparations for further development, since the compounds having the same mechanism of action will generally be the same (or nearly the same) compound. Using this information, duplication of development efforts can be avoided, providing for more rapid development of therapeutic agents from these sources.

While the discussion above emphasized screening 10 for inhibitors of bacterial growth, the invention is not limited to those embodiments. As indicated above, the methods of this invention are applicable to other cell types, therefore, other embodiments are within the following claims.

Claims

What we claim is:

1. A method for screening for a modulator of a biomolecule comprising the steps of:

- 5 a) growing a first cell type having an altered said biomolecule and a second cell type having a normal said biomolecule in a growth medium;
- b) contacting at least one said cell type with a potential modulator; and
- 10 c) determining the effect of the presence of said potential modulator by comparing a phenotypic sensor between said first and second cell types;
- wherein said first cell type has a conditional growth phenotype or said biomolecule has a partially crippled
- 15 function.

2. The method of claim 1, wherein said first and second cell types are each in contact with said potential modulator in a growth medium, and

wherein said phenotypic sensor differs to a greater

20 degree between said first and second cell types in the presence of a modulator of said biomolecule than in the absence of said modulator.

3. The method of claim 2, wherein said comparing a phenotypic sensor comprises comparing the

25 growth of said first and second cell types.

4. The method of claim 3, wherein said first and second cell types are separately in contact with said potential modulator.

5. The method of claim 4, wherein the growth of a plurality of said first cell types having different altered said biomolecules are compared to the growth of said second cell type.

6. The method of claim 1, wherein said second cell type is grown in the presence of said potential modulator and said first cell type in the absence of said modulator, and

said comparing comprises determining whether said phenotypic sensor is the same for said second cell type as for said first cell type.

15 7. The method of claim 6, wherein said first and second cell types are grown under a plurality of different growth conditions.

8. The method of claim 7, wherein said plurality of different growth conditions comprises a plurality of different growth media differing in their carbon sources.

9. The method of claim 6, wherein a plurality of said first cell types are grown, and said comparing comprises determining whether said phenotypic sensor is the same for said second cell type as for one or more of said first cell types.

10. The method of claim 1, wherein said conditional growth phenotype is a temperature sensitive phenotype.

11. The method of claim 1, wherein the presence of a modulator is determined by comparing the effect of said modulator on a plurality of different phenotypic sensors.

12. A method of screening for a modulator of a biomolecule, comprising the steps of:

10 a) growing a plurality of first cell types having altered biomolecules and a second cell type having normal said biomolecules;

b) contacting said plurality of first cell types and/or said second cell type with a potential modulator;
15 and

c) determining the effect of the presence of said potential modulator by comparing a phenotypic sensor between said plurality of first cell types and second cell type.

20 13. The method of claim 12, wherein said first and second cell types are each in contact with said potential modulator in a growth medium, and

wherein said phenotypic sensor differs to a greater degree between said first and second cell types in the presence of a modulator of said biomolecule than in the
25 absence of said modulator.

14. The method of claim 13, wherein said comparing a phenotypic sensor comprises comparing the growth of said first and second cell types.

15. The method of claim 11, wherein said second cell type is grown in the presence of said potential modulator and said plurality of first cell types in the absence of said modulator, and

said comparing comprises determining whether said phenotypic sensor is the same for said second cell type as for any of said first cell types.

16. The method of claim 15, wherein said first and second cell types are grown under a plurality of different growth conditions.

17. The method of claim 16, wherein said plurality of different growth conditions comprises a plurality of different growth media differing in their carbon sources.

18. The method of claim 12, wherein at least one said first cell type has a conditional growth phenotype.

19. The method of claim 18, wherein said conditional growth phenotype is a temperature sensitive phenotype.

20. The method of any of claims 12-19, further comprising simultaneously determining the mechanism of action of a modulator of a biomolecule,

comprising comparing the pattern of growth of said plurality of first cell types in the presence of said modulator with the pattern of growth in the presence of a modulator having a known mechanism of action.

21. The method of any of claims 12-19, further comprising simultaneously determining the mechanism of action of a modulator of a biomolecule,

comprising comparing the effects of said modulator on one or more phenotypic sensors in said second cell type with the pattern of effects of a modulator having a known mechanism of action on said phenotypic sensors in said plurality of first cell types.

22. The method of any of claims 1-19, wherein at least one of said first cell types is a microbe.

23. The method of claim 22, wherein at least one said microbe is a bacterial strain.

20 24. The method of claim 23, wherein said bacterial strain is selected from the group consisting of the Staphylococci, the Pseudomonads, the Enterococci, the Enterobacteriaceae, and the Streptococci.

25. The method of claim 24, wherein said bacterial strain is a *Staphylococcus aureus* or *Salmonella typhimurium* strain.

26. The method of claim 22, wherein said microbe is a fungus.

27. The method of claim 26, wherein said fungus is selected from the group consisting of *Saccharomyces cerevisiae*, *Aspergillus niger*, *Candida albicans*, and *Candida glabrata*.

28. The method of claim 21, wherein at least one of said first cell types is a mammalian cancer cell.

29. The method of any of claims 1-19, wherein at least one alteration of a biomolecule is a defect in said biomolecule which lowers the activity of said molecule.

30. The method of any of claims 1-19, wherein growth is measured by an indirect method.

15 31. The method of any of claims 1-19, wherein at least one said altered biomolecule is overexpressed with respect to the wild type cell.

32. The method of any of claims 1-19, wherein at least one said altered biomolecule is underexpressed 20 with respect to the wild type cell.

33. A method of screening for a modulator of a biomolecule comprising simultaneously determining the effects of said modulator in a plurality of assays,

wherein said plurality of assays characterizes a plurality of different properties of said modulator.

34. A method for evaluating a modulator of a biomolecule, comprising simultaneously determining the effects of said modulator in a plurality of assays, wherein said plurality of assays characterizes a plurality of different properties of said modulator.

35. The method of claim 33 or 34, wherein at least one said assay comprises determining the effect of said modulator on the growth of a cell type having a conditional growth phenotype.

36. The method of claim 33 or 34, further comprising determining the growth of a plurality of cell types having conditional growth phenotypes in the presence of said modulator.

37. The method of claim 33 or 34, wherein at least one said assay comprises determining the serum binding of said modulator in mammalian serum.

38. The method of claim 33 or 34, wherein at least one said assay comprises determining the serum inactivation of said modulator in mammalian serum.

39. The method of claim 33 or 34, wherein at least one said assay comprises determining the stability of said modulator in a solution or suspension.

40. The method of claim 33 or 34, wherein at least one said assay comprises determining the frequency or mechanism of development of resistance to said modulator.

5 41. The method of claim 33 or 34, wherein at least one said assay comprises determining the solubility of said modulator in a solvent or solution.

42. The method of claim 33 or 34, wherein at least one said assay comprises determining a cellular
10 toxicity of said modulator.

43. The method of claim 42, further comprising determining a molecular toxicology profile of said modulator, wherein the pattern of toxicological effects of said modulator in a plurality of cell types is
15 compared to the pattern of toxicological effects of one or more other compounds.

44. The method of claim 43, wherein comparing the pattern of toxicological effects of said modulator in a plurality of cell types to the pattern of toxicological
20 effects of one or more other compounds further comprises characterizing the molecular interactions of said modulator.

45. The method of claim 43, wherein said plurality of cell types comprises a plurality of
25 bacterial strains.

46. The method of claim 45, wherein said plurality of bacterial strains comprises *Salmonella typhimurium* or *Staphylococcus aureus* strains.

47. The method of claim 43, wherein said plurality of cell types comprises a plurality of fungal strains.

48. The method of claim 44, wherein said plurality of cell types comprises a plurality of bacterial strains.

10 49. The method of claim 48, wherein said plurality of bacterial strains comprises *Salmonella typhimurium* or *Staphylococcus aureus* strains.

50. The method of claim 44, wherein said plurality of cell types comprises a plurality of fungal
15 strains.

51. The method of claim 33 or 34, wherein said plurality of assays comprises determining the breadth of spectrum of said modulator.

52. The method of claim 33 or 34, wherein at
20 least one said assay comprises determining the cellular target of said modulator.

53. The method of claim 52, wherein said determining the cellular target of said modulator

comprises comparing the effect of said modulator on the growth of a plurality of cell types.

54. The method of claim 53, wherein said plurality of cell types comprises a plurality of cell types having conditional growth phenotypes.

55. The method of claim 54, wherein at least one said conditional growth phenotype is a temperature sensitive phenotype.

56. The method of claim 52, wherein said determining the cellular target comprises comparing a plurality of phenotypic sensors in a plurality of cell types.

57. The method of claim 33 or 34, wherein at least one said cell type is a microbe.

15 58. The method of claim 57, wherein said microbe is a bacterial strain.

59. The method of claim 58, wherein said bacterial strain is selected from the group consisting of the Staphylococci, the Pseudomonads, the Enterococci, 20 the Enterobacteriaceae, and the Streptococci.

60. The method of claim 57, wherein said microbe is a fungus.

61. The method of claim 60, wherein said fungus is selected from the group consisting of *Saccharomyces cerevisiae*, *Aspergillus niger*, *Candida albicans*, and *Candida glabrata*.

5 62. The method of claim 33 or 34, wherein at least one of said first cell types is a mammalian cancer cell.

63. A method for determining a phenotypic profile of a cell type comprising the step of determining
10 the effect of a modulator or an altered biomolecule on a plurality of phenotypic sensors of said cell type.

64. The method of claim 63, further comprising a plurality of different assays which characterize a plurality of different properties of said cell type.

15 65. A method for characterizing the mechanism of action of a modulator of a biomolecule, comprising the steps of:

- a) growing a plurality of cell types having different altered biomolecules in the presence of said modulator;
- 20 b) determining the effect of said modulator on a phenotypic sensor of said cell types; and
- c) comparing the effect of said modulator on said phenotypic sensor to the effect of a known modulator on said phenotypic sensor.

66. The method of claim 65, further comprising comparing the effects of said modulator on a plurality of phenotypic sensors.

67. The method of claim 65, comprising the 5 steps of

determining the pattern of growth of a plurality of cell types having different altered biomolecules in the presence of said modulator, and

comparing the pattern of growth of said plurality of 10 cell types in the presence of said modulator with the pattern of growth of said plurality of cell types in the presence of a known modulator.

68. The method of claim 65, wherein the mechanism of action of said known modulator is known.

15 69. The method of claim 65, wherein at least one said cell type is a conditional growth mutant.

70. The method of claim 69, wherein said conditional growth mutant is a temperature sensitive mutant.

20 71. The method of claim 69, wherein said conditional growth mutant is grown under semi-permissive conditions.

72. The method of claim 65, wherein said characterizing comprises identifying the cellular target 25 of said modulator.

73. A method for characterizing the mechanism of action of a modulator of a biomolecule, comprising the steps of:

- a) contacting a first cell type with a modulator;
 - 5 b) determining the effect of said modulator on a phenotypic sensor in first cell type; and
 - c) comparing said effects of said modulator on a phenotypic sensor in said first cell type with the effects of altered biomolecules on said phenotypic sensor
 - 10 in a plurality of cell types having different said altered biomolecules,
- wherein said first cell type has normal said biomolecules.

74. The method of claim 73, further comprising

15 comparing the effects of said modulator on a plurality of phenotypic sensors.

75. The method of claim 73, wherein at least one said cell type is a conditional growth mutant.

76. The method of claim 75, wherein said

20 conditional growth mutant is a temperature sensitive mutant.

77. The method of claim 75, wherein said conditional growth mutant is grown under semi-permissive conditions.

78. The method of claim 73, wherein said characterizing comprises identifying the cellular target of said modulator.

79. The method of any of claims 63-78, wherein one or more said cell types are microbes.

80. The method of claim 79, wherein one or more said microbes are bacterial strains.

81. The method of claim 80, wherein at least one of said bacterial stains is selected from the group consisting of the Staphylococci, the Pseudomonads, the Enterococci, the Enterobacteriaceae, and the Streptococci.

82. The method of claim 79, wherein one or more said microbes are fungi.

83. The method of claim 82, wherein said fungus is selected from the group consisting of *Saccharomyces cerevisiae*, *Aspergillus niger*, *Candida albicans*, and *Candida glabrata*.

84. The method of any of claims 63-78, wherein at least one of said first cell types is a mammalian cancer cell.

85. A method for characterizing a natural products preparation, comprising the steps of

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- a) growing a plurality of cell types having different altered biomolecules in a growth medium;
- b) contacting said plurality of cell types with a natural products preparation; and
- 5 c) determining the effect of the presence of said natural products preparation by comparing a phenotypic sensor between said plurality of cell types in the presence of said natural products preparation and in the presence of a second natural products preparation or a
- 10 known modulator of a biomolecule.

86. A method for characterizing a natural products preparation, comprising the steps of

- a) determining the pattern of effects on one or more phenotypic sensors for a plurality of cell types having
- 15 different altered biomolecules; and
- b) comparing the effects of a natural products preparation on said one or more phenotypic sensors in a cell type having normal said biomolecules with said pattern.

20 87. The method of claim 86, wherein said pattern of effects is a pattern of carbon source utilization.

88. The method of claim 85, wherein at least one said cell type has a conditional growth phenotype.

25 89. The method of claim 88, wherein said conditional growth phenotype is a temperature sensitive phenotype.

90. The method of any of claims 85-89, wherein at least one of said plurality of cell types is a microbe.

91. The method of claim 90, wherein at least one said microbe is a bacterial strain.

92. The method of claim 91, wherein said bacterial strain is selected from the group consisting of the Staphylococci, the Pseudomonads, the Enterococci, the Enterobacteriaceae, and the Streptococci.

10 93. The method of claim 90, wherein at least one said microbe is a fungus.

94. The method of claim 85 or 86, wherein said characterizing comprises determining whether said first natural product preparation inhibits a different genetic
15 target than said second natural products preparation or said known modulator of a biomolecule.

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FIG. 1.

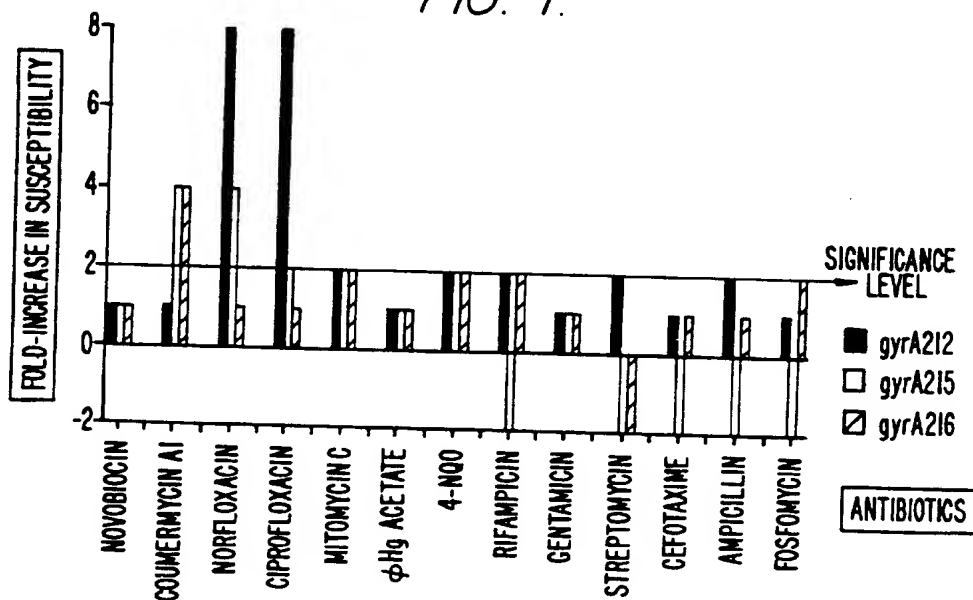
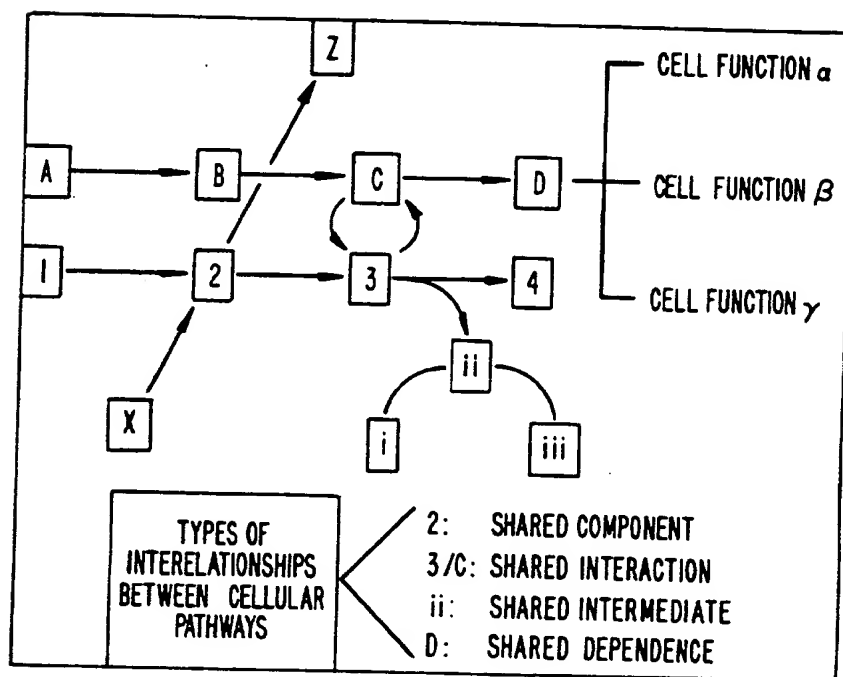


FIG. 3.



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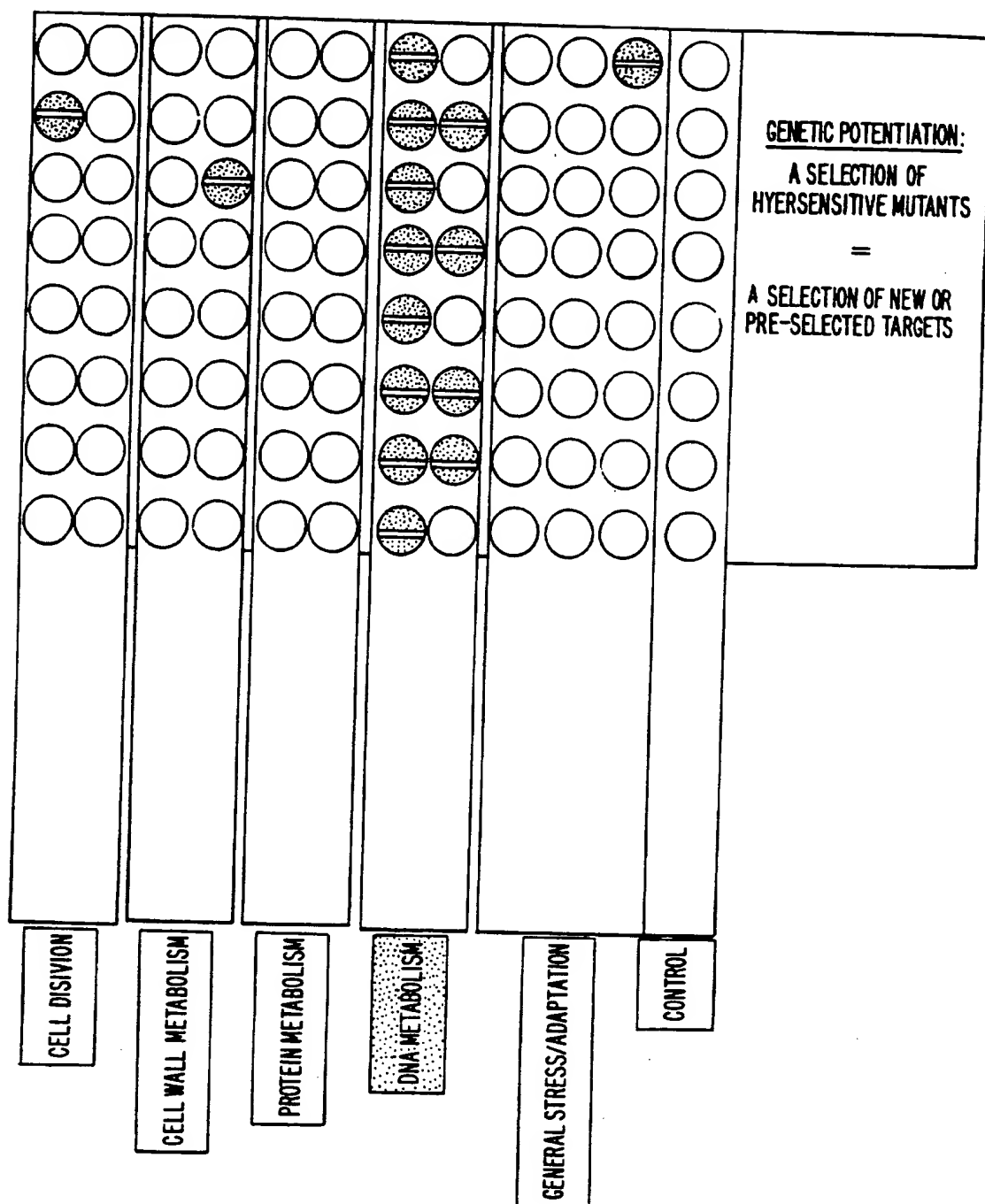
FIG. 2.

	Gyrase inhibitors				DNA/RNA metabolism				Protein metabolism			Cell wall inhibitors		
	Nov	Cou	Cipro	Nor	MitoC	φHg	NQO	Rif	Gen	Strep	Phen	Cefo	Amp	Fosfo
5155 dndE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7393 gyrA216	-	4	-	-	-	-	-	-	-	-	-	-	-	4
7392 gyrA215	-	4	-	≥4	-	-	-	-	-	-	-	-	-	-
7533 gyrA212	-	-	8	≥8	-	-	-	-	-	-	-	-	-	-
7784 parC	-	-	-	-	-	-	-	-	-	-	ND	-	-	-
5026 clm?	-	4	-	-	-	4	-	-	-	-	-	-	-	+8
5206 parE	-	-	-	-	-	4	-	-	-	-	4	-	-	-
8041 parE	-	-	-	-	-	4	-	-	-	-	4	-	-	-
5174 parF	≥4	16	-	+4	-	16	-	ND	-	ND	4	ND	ND	-
5178 parF	≥2	≥64	-	-	8	32	4	-	8	8	-	-	-	-
7818 parF	≥2	4-16	-	-	-	-	-	-	4-8	4	-	-	-	-
7109 clm?	-	-	-	-	-	4	-	-	-	-	-	-	-	-
5045 murB	-	-	-	-	-	16	-	-	-	-	-	-	-	-
7583 Round	-	-	-	-	-	4	-	-	-	-	-	-	-	-
7587 dapA	-	-	-	-	-	32	-	-	-	-	-	-	-	-
5119 murCEFG	≥32	≥64	8	-	64	64	8	-	4	-	32	≥8	2-4	-
5091 Thyn	≥64	≥64	-	-	8	4	4	-	4	-	32	8	32	-
7585 Oxd	-	-	-	-	-	-	-	-	-	-	-	4	8	-
5208 flsH	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7141 Filom	-	-	-	-	-	-	-	-	-	4	-	-	-	-
5052 Filom	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5051 Filom	-	-	-	-	16	-	64	4	-	-	-	-	-	-
5041 UV-	-	-	-	-	-	16	4	4	-	-	-	-	-	+4
5066 UV-	-	4	-	+4	4	-	32	4	-	-	-	-	-	+32
5258 clm?	-	-	-	-	4	16	16	4	8	-	4	-	-	+8
														4

"-" INDICATES THAT THERE WAS NO SIGNIFICANT DIFFERENCE WITH THE WILD TYPE PARENT STRAIN. "ND": NOT DETERMINED.

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FIG. 4.



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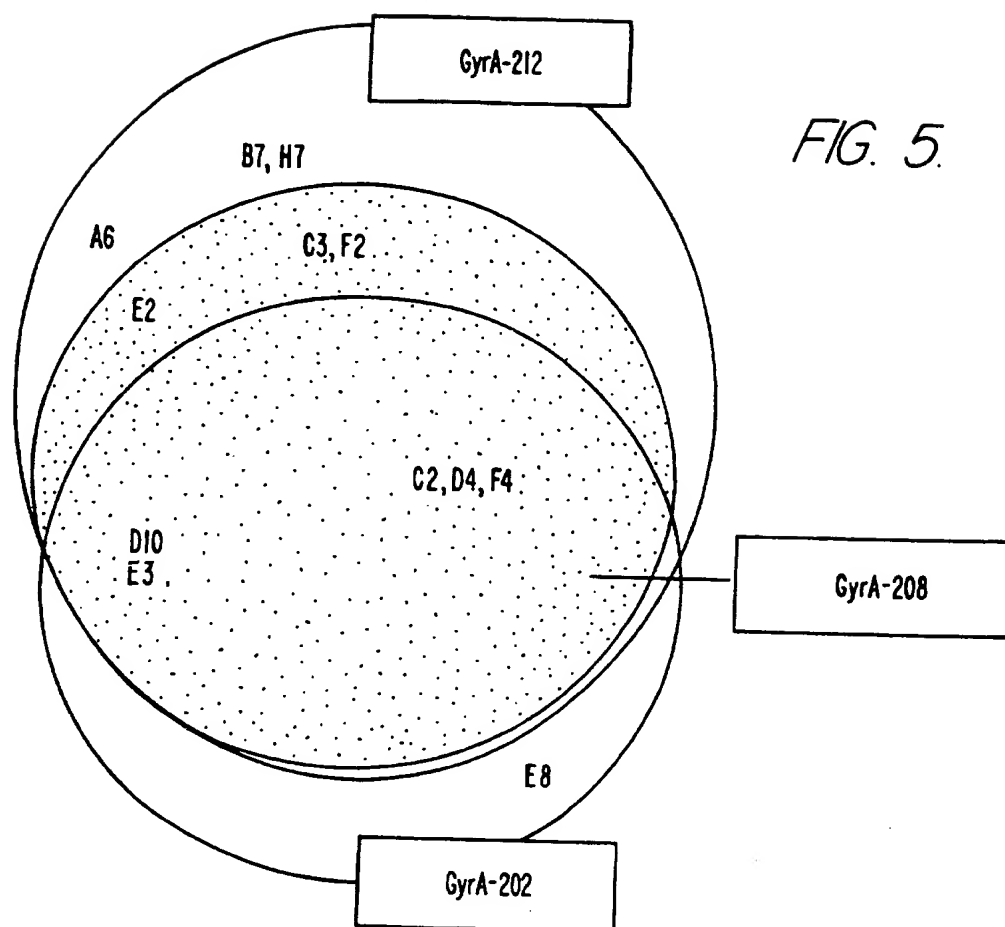
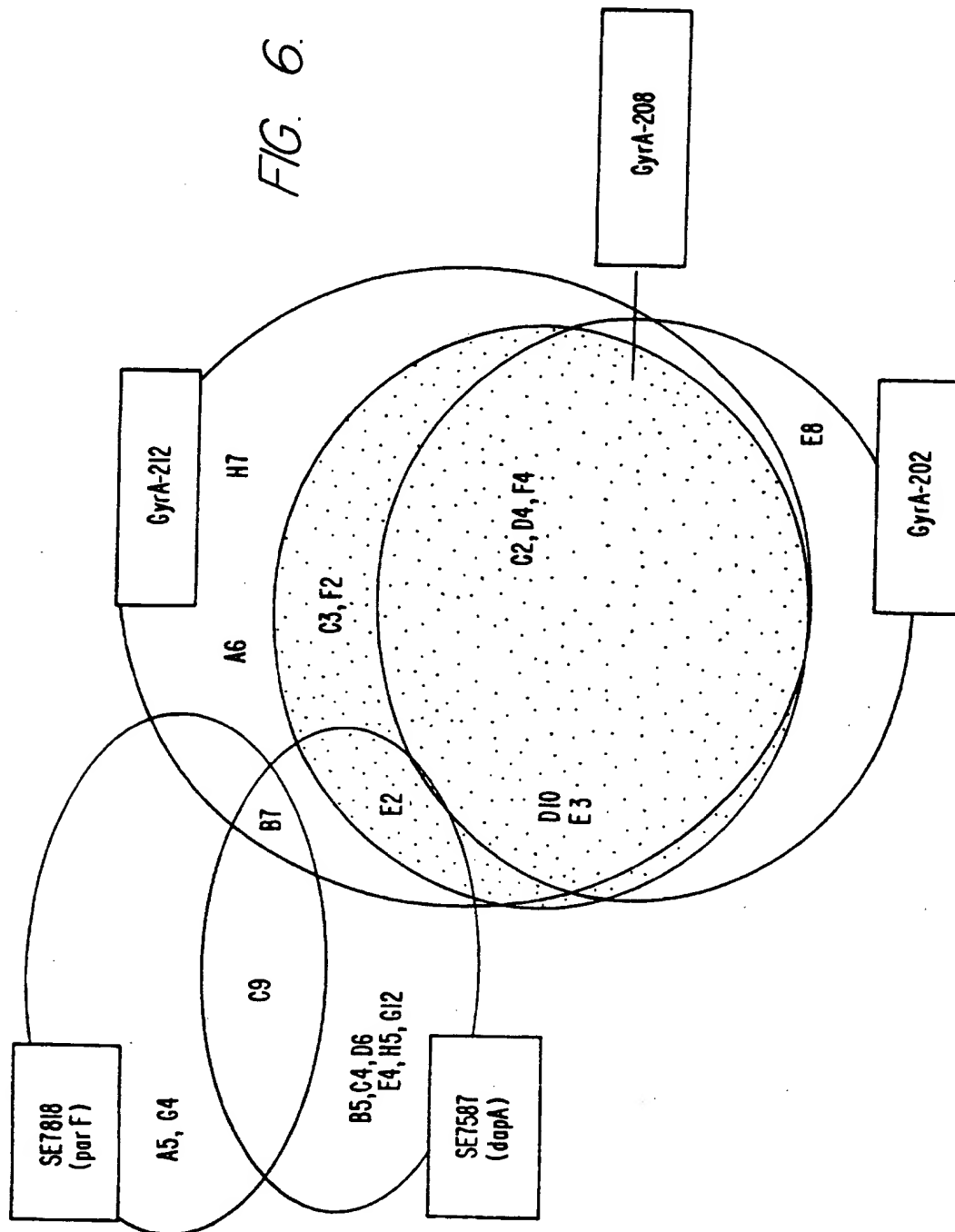


FIG. 5.

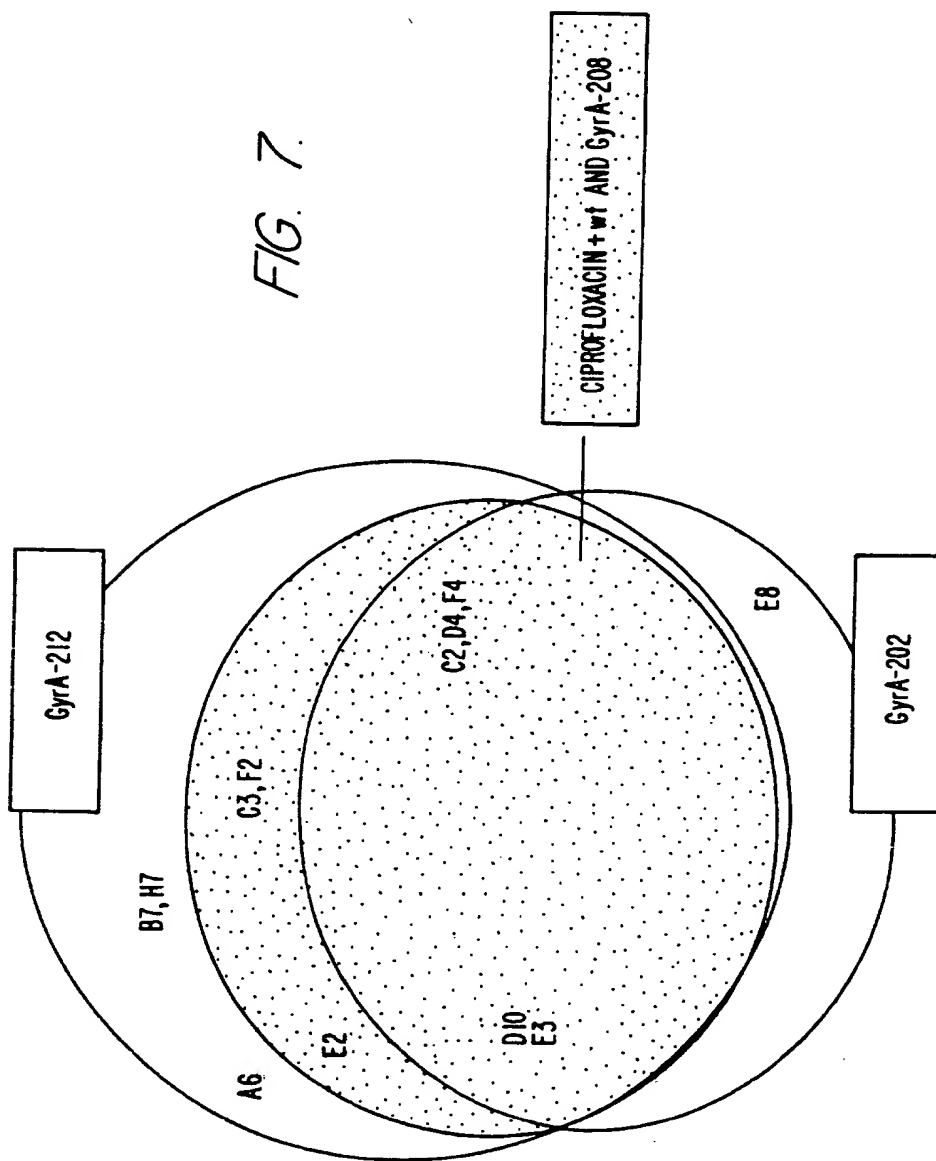
5/17

FIG. 6.



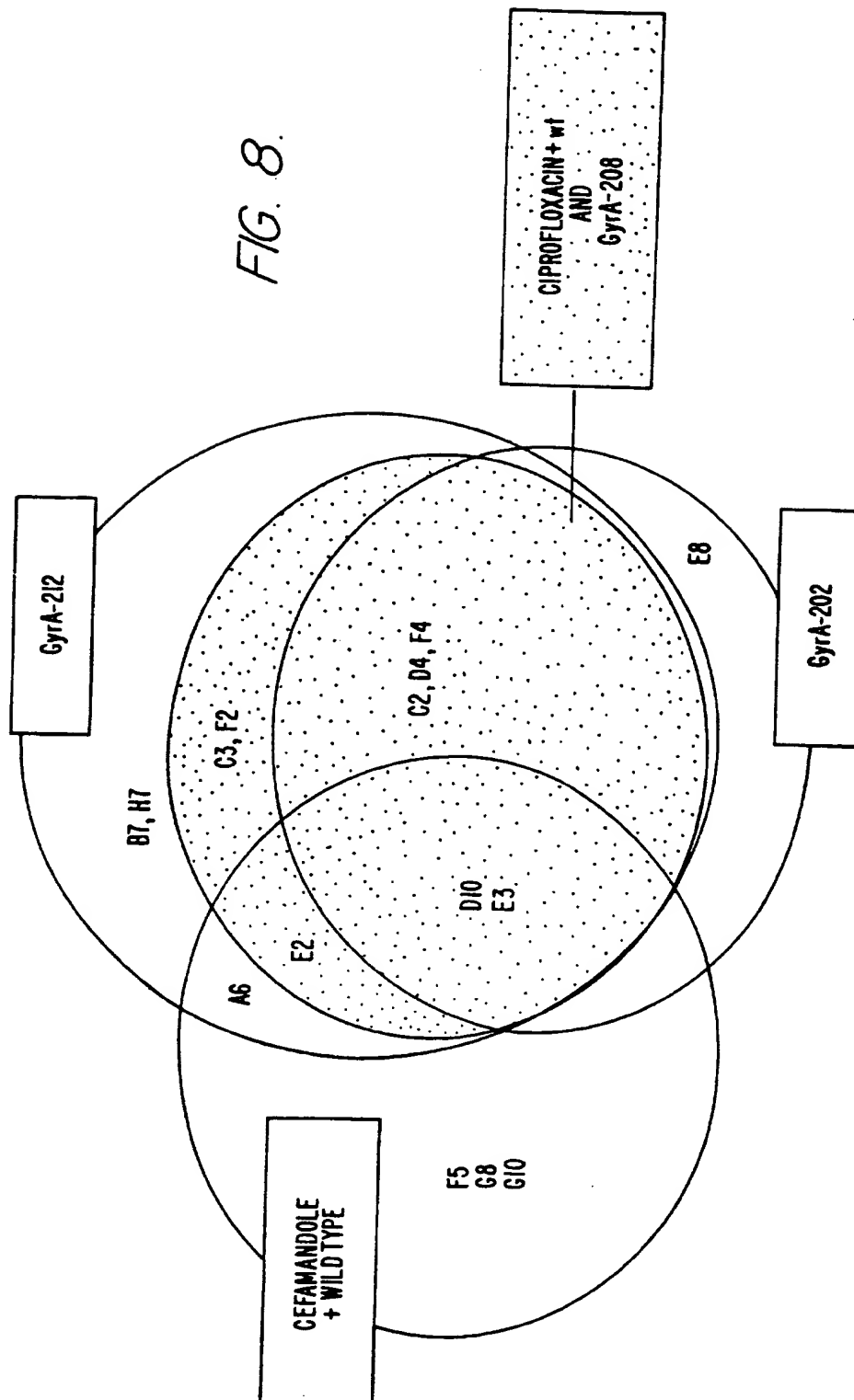
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FIG. 7.



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FIG. 8.



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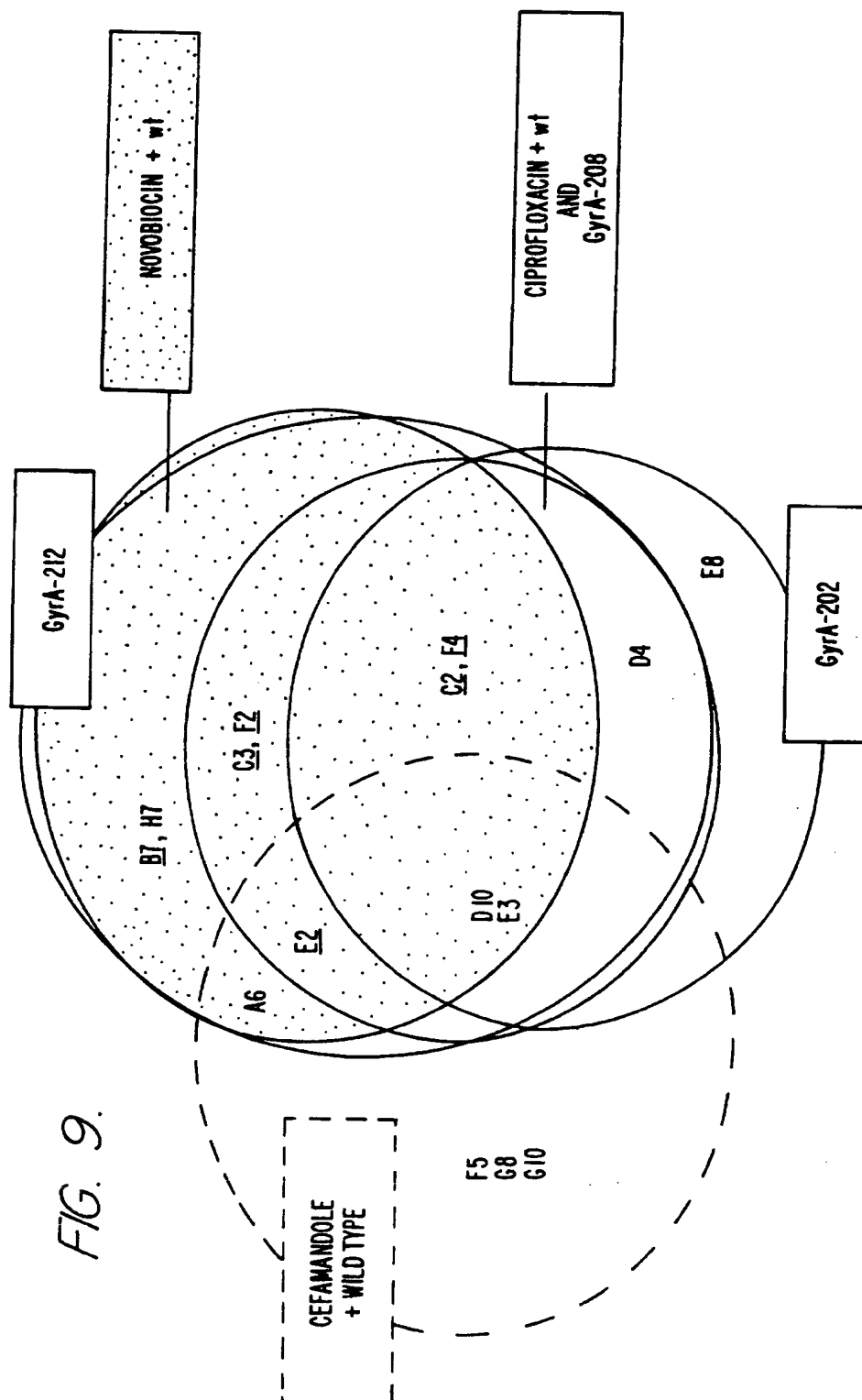


FIG. 9.

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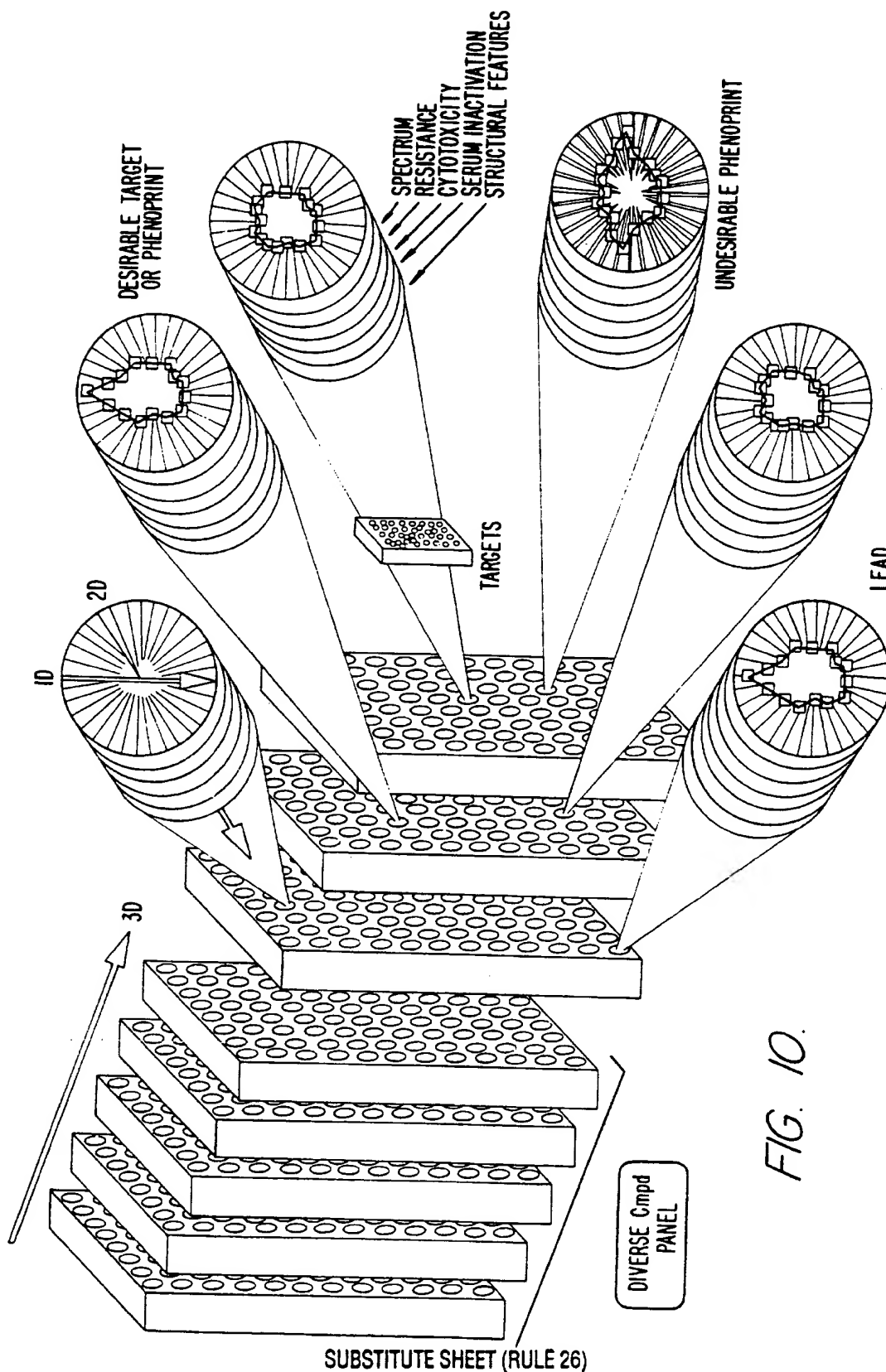


FIG. 10.

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FIG. 11.

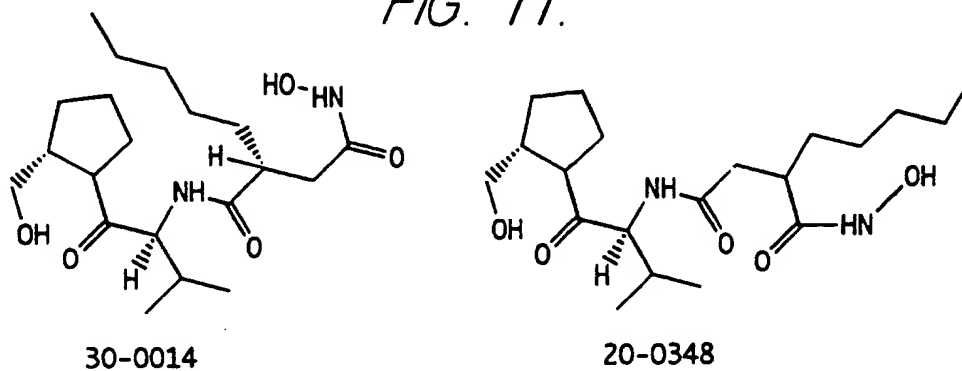
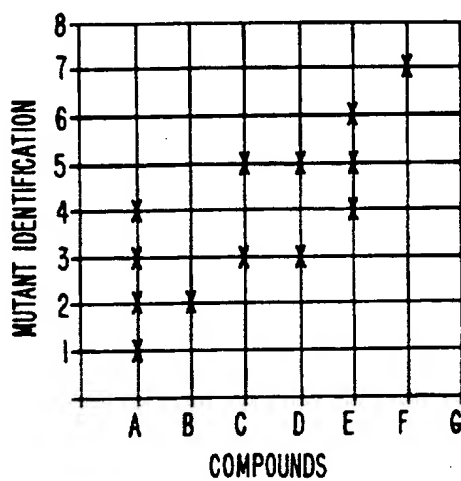


FIG. 14.



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FIG. 12a.

NT	20-0157	10-0167	50-0116	20-0204	20-0860	20-0123	10-0287	20-0045	10-0373	10-0752	20-0197	30-0014	20-0348	10-0797	00-3775	00-9370	00-2002	00-0167
2																4	8	4
3																	8	
4																		
5																		
6																4-8		
8																		
10																		
12										16	≥ 4					ND	ND	ND
14																4	4	4
15																≥ 4		
16																		
18																4	16	4
16												4	4				8	8
22																	8	4
23																4	4	8
27																		8
28																4	4	4
29									≥ 8						4-8	4	4	4
33															≥ 2	16-32	4-8	4-8
36												4	8				8	8
42						8		8		≥ 2		4	8				4	4
47												4	4			8		
50											8	32	64	8			8	8
51																		
52																	8	8

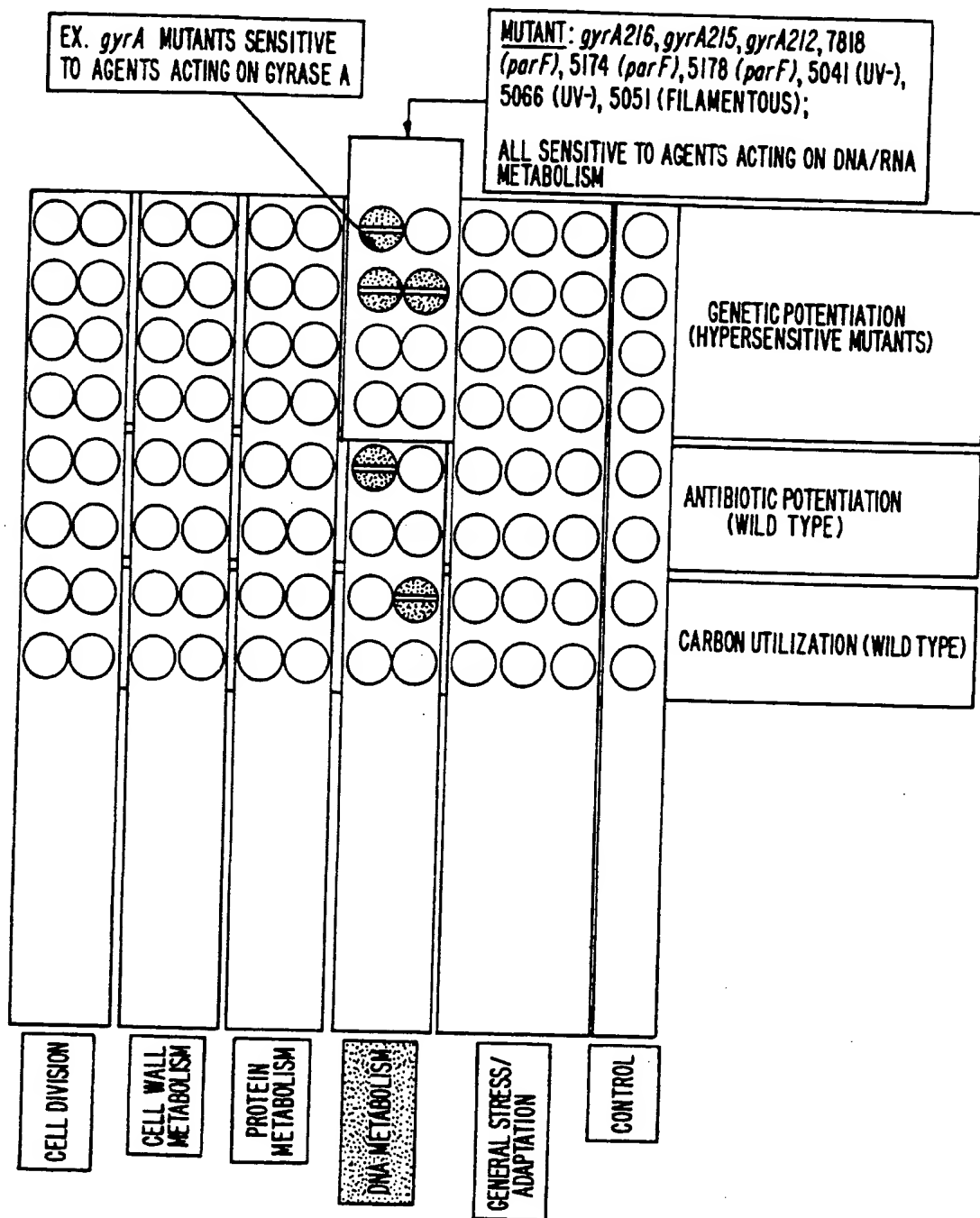
FIG. 12b.

SUBSTITUTE SHEET (RULE 26)

*ND: NO DATA AVAILABLE: BLANK BOXES SHOW NO SIGNIFICANT DIFFERENCE IN MIC FROM THE WILD-TYPE STRAIN (SIGNIFICANCE LEVEL $>+/-$ 2 FOLD).

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FIG. 13.



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FIG. 15.

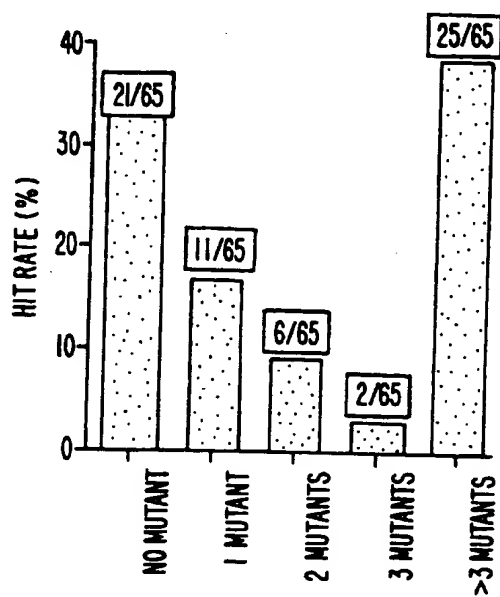
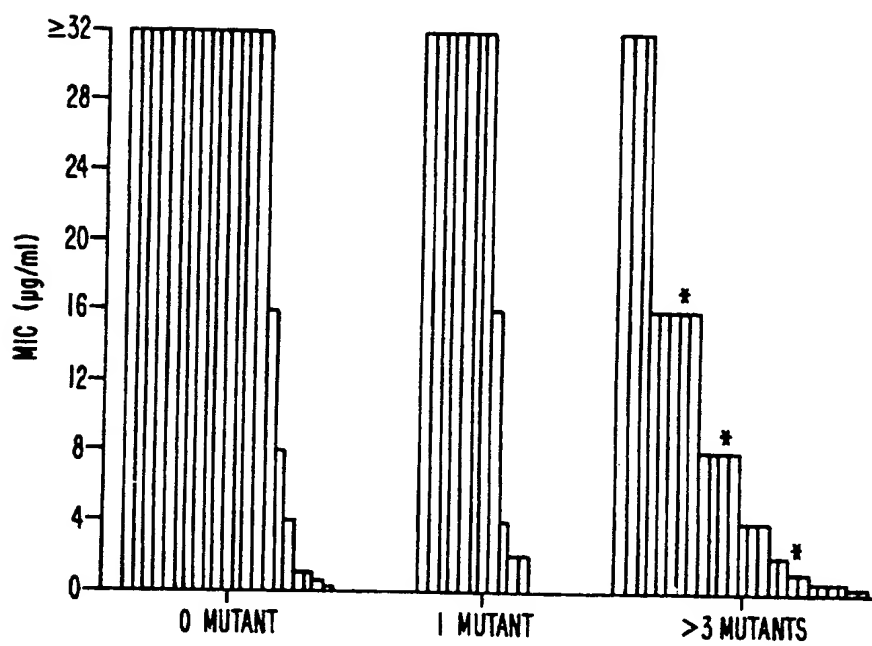


FIG. 16.



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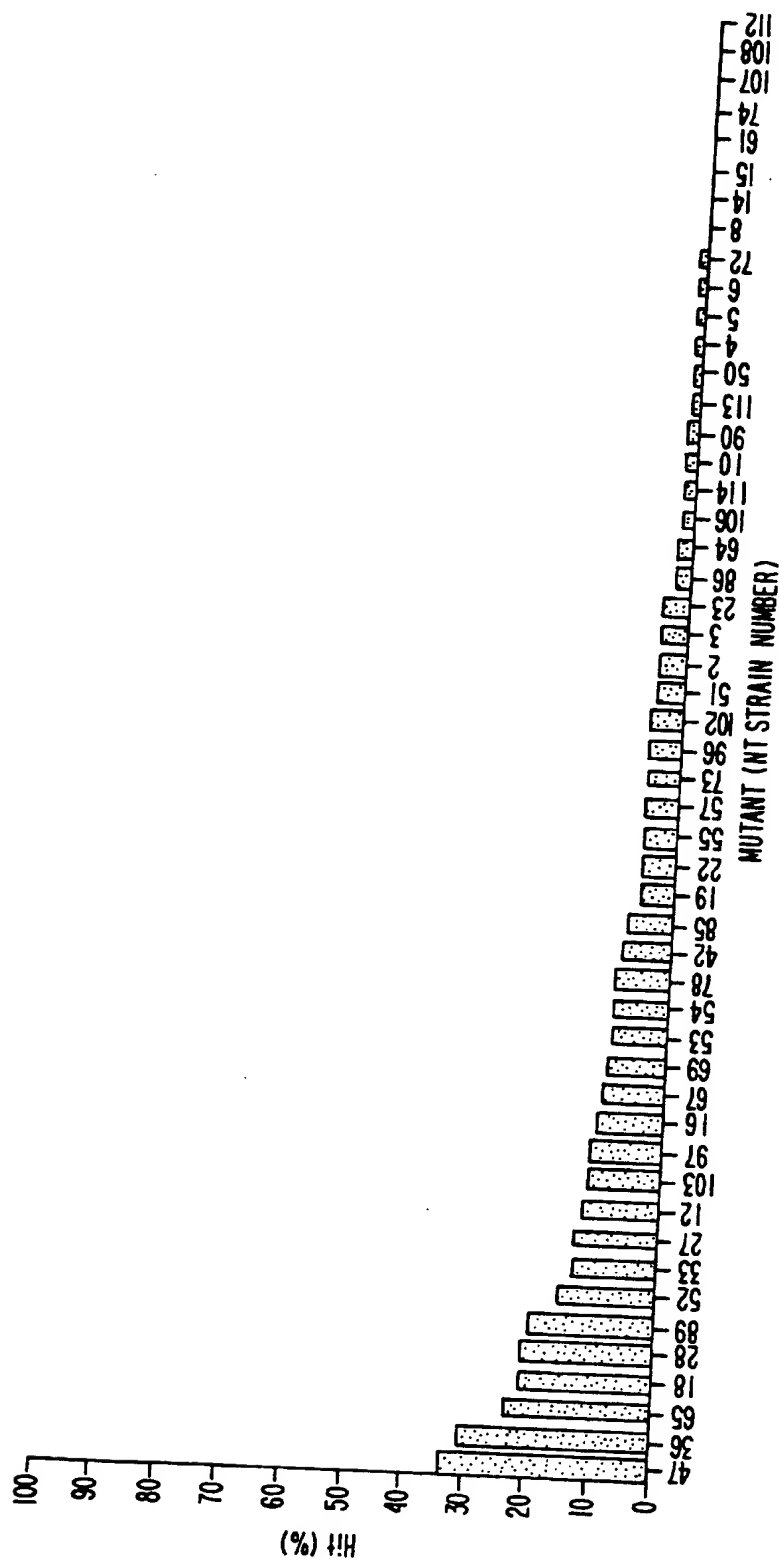


FIG. 17.

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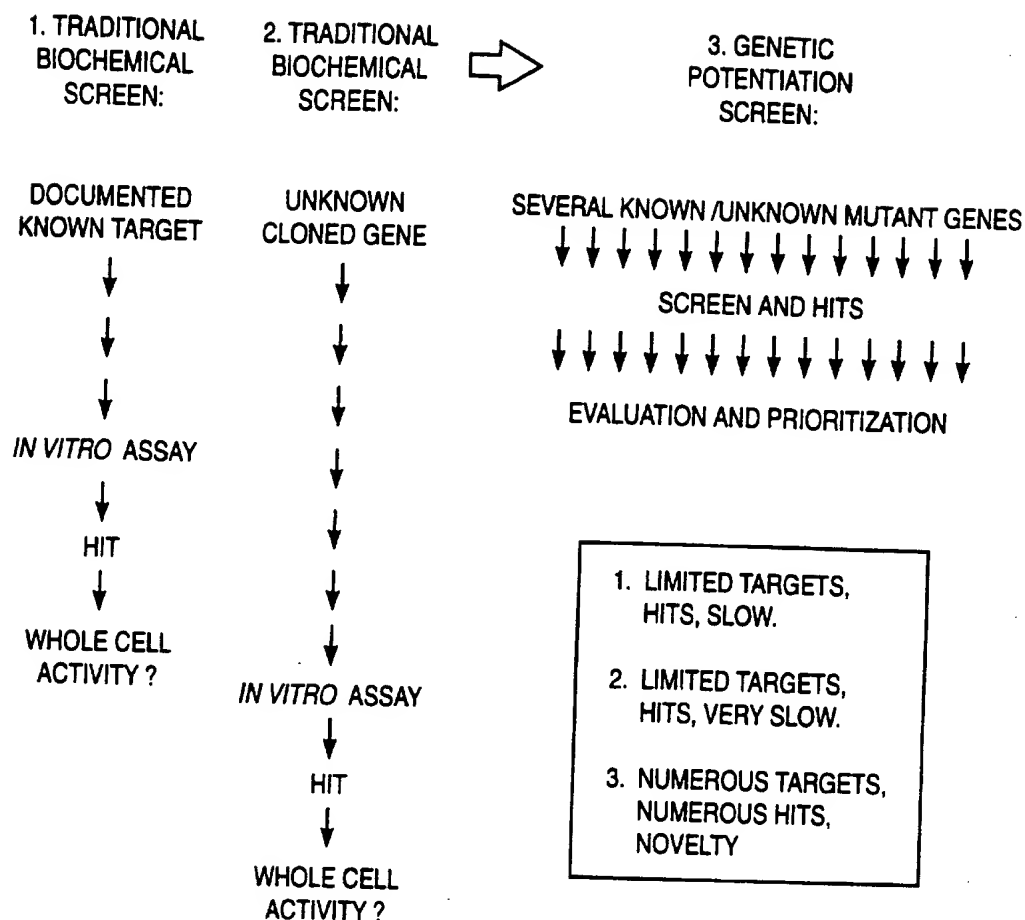


FIG. 18.

FIG. 19.

Relevant Genotype	Phenotype
ts/hypersens	No growth at high temperature
dom	No growth (not viable)
ts/hypersens dom	No growth (not viable)
ts/hypersens dom	Growth at high temperature No growth at low temperature

SUBSTITUTE SHEET (RULE 26)

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FIG. 20

Goal:	Concept, Approach or Discriminator:
Screening and identification of modulators of biomolecules	Phenotypic differences between two cell types
	Plurality of cell types
	Plurality of assays
Determining the mechanism of action of modulators	Pattern of growth of a plurality of cell types
	Phenotypic profiles of a plurality of cell types
Characterization of the mechanism of action of modulators	Plurality of cell types
	Plurality of phenotypic sensors
Evaluating a modulator	Plurality of assays
Phenotypic characterization of cell types	Plurality of phenotypic sensors
	Plurality of assays
Characterization of natural products preparation	Plurality of cell types
	Pattern of effects on cell types
Distinguishing natural products preparations	Plurality of cell types
	Pattern of effects on cell types

INTERNATIONAL SEARCH REPORT

National Application No
PCT/US 96/00916

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/02 //C12Q1/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,95 23235 (MYCO PHARMACEUTICALS INC) 31 August 1995 see claim 1	1-4
P,A	--- EP,A,0 644 268 (AMERICAN CYANAMID CO) 22 March 1995 see the whole document	1
P,A	--- WO,A,95 06132 (MYCO PHARMACEUTICALS INC ;UNIV GEORGIA (US)) 2 March 1995 --- -/--	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

15 May 1996

Date of mailing of the international search report

11.06.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Hoekstra, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/00916

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>THE JOURNAL OF ANTIBIOTICS, vol. 39, no. 7, 1986, page 994-1000 XP002003099 NUMATA, K. ET AL.: "Isolation of an aminoglycoside hypersensitive mutant and its application in screening" cited in the application see the whole document ---</p>	1
A	<p>THE JOURNAL OF ANTIBIOTICS, vol. 41, no. 6, 1988, pages 803-806, XP002003100 KAMAGOSHIRA, T. AND TAKEGATA, S. : "A screening method for cell wall inhibitors using a D-cycloserine hypersensitive mutant" cited in the application see the whole document -----</p>	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/00916

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9523235	31-08-95	NONE	
EP-A-0644268	22-03-95	NONE	
WO-A-9506132	02-03-95	NONE	